Origine embryonnaire des cellules souches neurales adultes du pallium de poisson zèbre

Lara Dirian

Ecole Doctorale BIOSIGNE (419)

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THESE DE DOCTORAT

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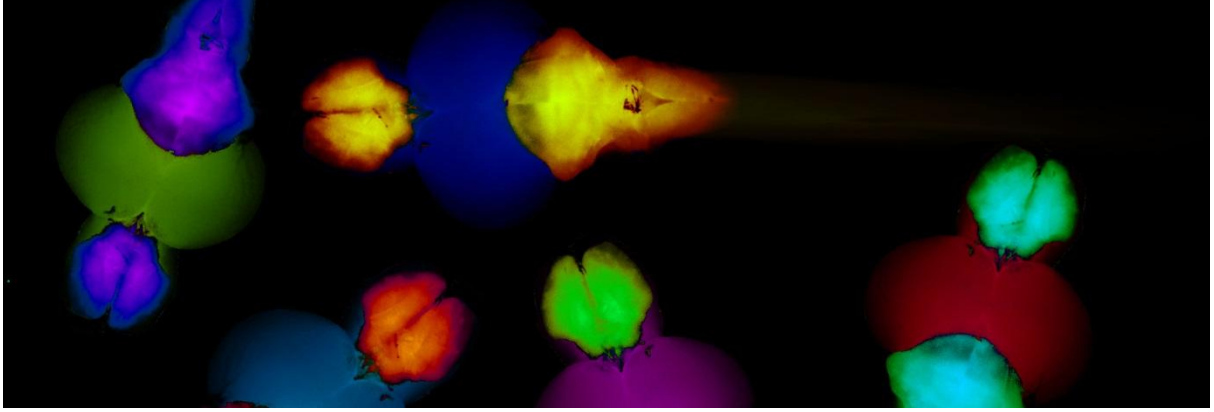

Soutenue le 13 novembre 2014

Membres du jury:

Dr. Laure BALLY-CUIF	Directrice de thèse
Dr. Isabelle FOUCHER	Co-encadrante
Pr. Jonathan CLARKE	Rapporteur
Dr. Alessandra PIERANI	Rapportrice
Dr. Cédric MAURANGE	Examinateur
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A ma famille,

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RESUME

Les cellules souches neurales adultes (aNSCs) sont définies par des fonctions d'auto-renouvellement et de multipotence qui leur permettent de générer dans le cerveau adulte tant des neurones que des cellules gliales. Contrairement aux mammifères, le cerveau de poisson zèbre présente de nombreuses zones de neurogenèse adulte dont la plus caractérisée est la zone ventriculaire du pallium. Elle est composée de cellules de glies radiaires qui font office de aNSCs dans cette partie du cerveau. Quels progéniteurs neuraux embryonnaires sont sélectionnés pour être à l'origine de ces aNSCs reste mal connu. Ce travail a pour objectif de déterminer la contribution relative de deux populations de progéniteurs neuraux embryonnaires, les "clusters proneuraux" (impliqués dans la neurogenèse embryonnaire) et les "pools de progéniteurs" (caractérisés par une neurogenèse tardive), dans la formation des aNSCs du pallium de poisson zèbre.

Dans un premier temps, à l'aide de techniques génétiques de lignage cellulaire, nous avons pu identifier la population de progéniteurs neuraux embryonnaires à l'origine d'une sous-population des aNSCs située dans la partie dorso-médiane du pallium. Des expériences de lignage utilisant la lignée de poisson zèbre *her4:ERT2CreERT2* combinées à des traitements inhibiteurs de la voie de signalisation Notch nous ont permis de déterminer que les progéniteurs neuraux donnant naissance aux aNSCs du pallium dorso-médian expriment le gène « *Enhancer of split » her4,* qui caractérise les "clusters proneuraux", ce dès des stades très précoces du développement.

Dans un second temps, des analyses clonales ainsi que des recombinaisons spatialement contrôlées par laser nous ont permis de mettre en évidence que les aNSCs de la partie latérale du pallium de poisson zèbre ne proviennent pas de progéniteurs embryonnaires exprimant *her4* et maintenus par la voie Notch, mais d'une population restreinte de cellules neuroépitheliales situées dans la plaque du toit du télencéphale embryonnaire. Ces cellules présentent des caractéristiques spécifiques des "pool de progéniteurs", à savoir l'expression de gènes *her* non-canoniques (dont l'expression n'est pas dépendante de la voie de signalisation Notch) tels que *her6* et *her9,* l'expression de ligands de voies de signalisation telles que Wnt, BMP et FGF, et une neurogenèse tardive. Elles génèrent progressivement, à partir du stade juvénile, une grande partie des aNSCs du pallium latéral. De plus, une partie de ces cellules neuroépitheliales persistent dans le pallium latéral postérieur chez l'adulte et continuent de former *de novo* des aNSCs dans cette région du cerveau.

Outre la vision globale que cette étude nous a permis d'avoir sur l'origine embryonnaire de la totalité des aNSCs du pallium de poisson zèbre, elle a aussi délivré des informations sur les étapes de maturation progressive des progéniteurs embryonnaires pour former les aNSCs, et les similitudes et divergences qui existent entre la population dorso-médiane et latérale à ce sujet. Enfin, en traçant les neurones issus des cellules souches à différents stades, cette étude a pour la première fois mis en évidence la formation progressive des compartiments neuronaux du pallium de poisson zèbre, et ainsi permis d'apprécier les homologies de ces compartiments avec les régions du pallium de souris.

SUMMARY

Adult neural stem cells (aNSCs) are defined by their self-renewal and multipotency, which allow them to generate both neurons and glial cells in the adult brain. Contrary to mammals, the zebrafish brain maintains numerous neurogenic zones in the adult, among which the most characterized is the pallial ventricular zone. It is composed of radial glial cells serving as aNSCs. Which embryonic neural progenitors are at the origin of these aNSCs is still unknown. This work aims to determine the relative contributions of two embryonic neural progenitor populations, the «proneural clusters» (involved in embryonic neurogenesis) and the « progenitor pools » (characterized by a delayed neurogenesis), to the formation of aNSCs in the zebrafish pallium.

First, using genetic lineage tracing techniques, we were able to identify the embryonic neural progenitor population at the origin of a subpopulation of aNSCs located in the dorso-medial part of the pallium. The *her4:ERT2CreERT2* transgenic driver line, combined with pharmacological treatments inhibiting the Notch signalling pathway, allowed showing that neural progenitors giving rise to dorso-medial pallial aNSCs express the « *Enhancer of split* » *her4* gene, specifically expressed in « proneural clusters » from very early stages of development.

As a second step, clonal analyses as well as spatially controlled recombinations by laser highlighted that aNSCs of the zebrafish lateral pallium do not derive from *her4*-positive embryonic progenitors maintained by the Notch pathway, but from a restricted population of neuroepithelial cells located in the embryonic telencephalic roof plate. These cells display « progenitor pool » specific features, as for instance the expression of non-canonical *her* genes (independent of Notch signalling) such as *her6* and *her9*, the expression of components of signalling pathways such as Wnt, BMP, FGF, and a late neurogenesis onset. These progenitors progressively generate, from juvenile stages, the vast majority of the aNSCs of the lateral pallium. Most interestingly, a small population of these neuroepithelial cells persists in the postero-lateral pallium at adult stage and keeps generating *de novo* aNSCs of this brain region.

In addition to identifying the origin of pallial aNSCs in the zebrafish, this study also delivers information on the progressive maturation steps that embryonic progenitors undergo to generate aNSCs, and highlights similarities and differencies existing between the dorso-medial and lateral progenitors. Finally, this work also permits tracing the neurons generated by stem cells at different stages. This reveals for the first time the progressive formation of the different zebrafish pallial compartements, and allows appreciating their homologies with the mouse pallial regions.

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ABBREVIATIONS

A/P	Antero-posterior
AC	Anterior commissure
AEP	Anterior entopeduncular area
aIPCs	Astrocytic intermediate progenitor cells
AIS	Anterior intraencephalic sulcus
AME	Anterior axial mesendoderm
AN	Ammonic neuroepithelium
ANB	Anterior neural border
ANR	Anterior neural ridge
aNSCs	Adult neural stem cells
AuA	Autonomic amygdala
AVE	Anterior visceral endoderm
bHLH	basic Helix-loop-Helix
BLA	Baso-lateral amygdala
BLBP	Brain-lipid-binding protein
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
Cer	Cerberus
CFP	Cyan fluorescent protein
CMZ	Ciliary marginal zone
CNS	Central nervous system
Co-P	Commissural plate
CP	Caudate putamen
CP	Cortical plate
CR	Cajal-Retzius
CSF	Cerebro-spinal fluid
Ctx	Isocortex
D/V	Dorso-ventral
Dc	Area dorsalis telencephali pars centralis (central zebrafish pallium)
Dd	Area dorsalis telencephali pars dorsalis (dorsal zebrafish pallium)
DGCs	Dentate granule cells
Di	Diencephalon

DKK1	Dickkopf-1
Dl	Area dorsalis telencephali pars lateralis (lateral zebrafish pallium)
Dll	Delta-like genes
Dm	Area dorsalis telencephali pars medialis (medial zebrafish pallium)
DMSO	Dimethyl sulfoxyde
DNA	Desoxyribonucleic acid
DNe	Dentate neuroepithelium
Dp	Area dorsalis telencephali pars posterior (posterior zebrafish pallium)
dpf	Days-post fertilization
DSL	Delta/Serrate/Lag2
DVR	Dorsal ventricular ridge
E(Spl)	Enhancer of split factors
EGF	Epidermal growth factor
ERT2	Estrogen receptor 2
EtOH	Ethanol
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FRP	Frizzled Related Proteins
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate transporter
GS	Glutamine synthase
GZ	Germinal zone
Ha	Habenula
Hem	Cortical Hem
Her	*Hairy* and *Enhancer of split* related genes
Hes	*Hairy* and *Enhancer of split* genes
Hsp	Heatshock promoter
IGF	Insulin-like growth factor
IP	Intermediate progenitors
IPCs	Intermediate progenitor cells
ISH	*in situ* hybridization
Kb	Kilo base
Lac-Z	beta-galactosidase gene
LGE	Lateral ganglionic eminence

LoxP	Locus of X-over P1
LP	Lateral pallium
MGE	Medial ganglionic eminence
MHB	Midbrain-hindbrain boundary
mpf	months-post fertilization
MZ	Marginal zone
NE	Neuroepithelial
NECD	Notch extracellular domain
NICD	Notch intracellular domain
nIPC	Neurogenic intermediate progenitor cell
NSCs	Neural Stem cells
OB	Olfactory bulb
OE	Olfactory epithelium
OHT	Hydroxy-tamoxifen
OPC	Oligodendrocytic progenitor cells
ORGCs	Outer radial glial cells
OSVZ	Outer-subventricular zone
Pal	Pallidum
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PGZ	Periventricular gray zone
PirCtx	Piriform cortex
PO	Preoptic area
POA	Anterior preoptic area
POC	Commissural preoptic area
POH	Preoptic-hypothalamic border
PP	Preplate
PSB	Pallial/subpallial boundary
RA	Retinoic acid
RBPJ	Recombining binding protein suppressor of hairless
RG	Radial glia
RGCs	Radial glial cells
RMS	Rostral migratory stream
RNA	Ribonucleic acid
Sep/Se	Septum
SEZ	Subependymal zone
SGZ	Subgranular zone

Shh	Sonic-hedgehog
SPl	Embryonic subplate
SPZ	Subpial zone
SVZ	Subventricular zone
TF	Transcription factor
TGFβ	Transforming growth factor β
TH	Thyroid hormone
TR	Thyroid hormone receptor
Ubi	Ubiquitin gene
VEGF	Vascular endothelial growth factor
VP	Ventral pallium
VZ	Ventricular zone
Y	Sulcus ypsiloniformis
YFP	Yellow fluorescent protein
ZLI	Zona Limitans Intrathalamica

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CHAPTER I: INTRODUCTION

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Preamble

The main aim of the present work was to identifiy the embryonic neural progenitor populations at the origin of the adult germinal zone (neural stem cell zone) of the dorsal telencephalon (pallium), using the zebrafish as a model.

For this, I combined different lineage tracing strategies to determine the progeny of distincts embryonic populations and appreciate their maturation from very early stages of development up to adult stage. Thus, in the first part of this introduction, I will review current knowledge on the patterning of the forebrain, ie. the brain subdivision hosting the telencephalon. Then, as I worked on neural progenitors, I will introduce how neurogenesis recruits neuroepithelial (NE) progenitors and/or radial glial cells (RGCs) toward the production of neurons during development and in the adult brain. In a third section, I will decribe the signals required to induce, maintain and control the fate of neural progenitors, again in the embryonic and in the adult brain contexts. Finally, the last part will describe the different hypotheses existing, at the onset of my PhD work, on the origin of adult neural stem cells (aNSCs), and the first information available on the embryonic populations contributing to aNSCs formation.

1 From the anterior neural plate to the adult dorsal telencephalon: development of the pallium

This first section will review current knowledge on the development of the most anterior part of the forebrain, the telencephalon, with a special focus on its dorsal subdivision, the pallium. I will start with describing the general organization of the adult telencephalon. Then, I will highlight the developmental processes that underlie forebrain development, from the induction of neural tissue during gastrulation to the specification of the different forebrain subdivisions: the telencephalon, the eyes and the hypothalamus (anterior forebrain), and the diencephalon including the pre-thalamus, the thalamus and the pre-tectum (posterior forebrain). In a third part, I will focus on the late development and maturation of the telencephalon, with a special focus on the pallium. Finally, I will discuss the similarities and potential difference that exist between the zebrafish and the mouse pallium in terms of homology and development.

1.1 Organization and functional characteristics of the adult telencephalon

Despite the amount of knowledge that emerged since the last century on the function, the organization, the development and the evolution of the brain, the telencephalon remains a fascinating structure. The complexity of its organization, its heterogeneous collection of neuronal cell types and the fact that it is considered as the region that hosts the consciousness and intelligence will make it a source of study still for a long time. During evolution, the telencephalon, and more generally the forebrain, is probably the brain region that has been submitted to the most important diversification concerning its size, neuronal composition, or connectivity. Despite this complex diversity, the general organization of the forebrain is conserved in all vertebrates, diversity occurring during morphogenesis and elaboration of the mature structures from a common ground plan. Early steps in CNS patterning are largely conserved, and studies primarily undertaken in chick, fish, frog, and mouse are beginning to unravel the mechanisms by which the forebrain is induced and patterned.

The telencephalon is composed of two territories, the pallium and the subpallium. In this first part, I will detail briefly the neuroanatomy and the function of these regions.

1.1.1 The pallium

The pallium is defined as the dorsal part of the telencephalon. At present, at least in tetrapods tetrapods (amphibians, sauropsids and mammals), the developing pallium is subdivided into four four different regions called medial, dorsal, lateral and ventral pallia, homologous as fields across across species. Nevertheless, the level of knowledge acquired on pallial subdivisions is extensively extensively different depending on the domain itself and on the species. Focusing on the mammalian mammalian pallium, several adult structures emerge from the dorsal telencephalon (Medina and Abellán, and Abellán, 2009)(

Figure 1).

We first can start with the **dorsal pallium,** generating the "**isocortex**" (or neocortex). It is the center of higher cognitive functions; it receives sensory inputs from environment and integrates them to generate the appropriate behaviors (Kandel et al., 2000). This brain structure is composed of six horizontal layers of specific neuronal subtypes organized following an inside-out sequence, with the latest-born neurons finally located in the most superficial layer. These layers compose 4 spatiallly distincts "primary" areas: the primary visual (V1), the somatosensory (S1), the auditory area (A1), which process information coming from the eye/retina (vision), the body (somatosensory), and inner ear/cochlea (audition), respectively, and the primary motor area (M1), which controls voluntary movements. They are connected to four specific nuclei in the thalamus, which receive modality-specific sensory information from peripheral sense organs or receptors and thus define the functional modality of the targeted primary cortical area of which they are specific (O'Leary and Sahara, 2008). The cortical territories have a specific size and are positioned at precise spatial coordinates relative to each other.

The second most important region, deriving from the **medial pallium**, is the **hippocampus**. The function of the hippocampus is to mediate spatial learning and memory mainly thanks to information transiting via the entorhinal cortex (Kandel et al., 2000). In all tetrapods, a hippocampal-like area produced in the medial pallium is devoted to this function (Medina and Abellán, 2009). This structure is composed of a single pyramidal cell layer. This layer, submitted to folding rearrangements during its development (see section1.3.4), is subdivided into fields called *cornu ammonis* (CA): CA1 and CA3 are the two major fields, separated by a small transitional field CA2. These fields are capped by the dentate gyrus, the third major area of the hippocampus (Khalaf-Nazzal and Francis, 2013)

According to recent proposals, the **lateral and ventral pallia** have to be considered as two different neuroanatomical domains of the telencephalon (Puelles et al., 2000), even though each of them generates parts of the olfactory cortex (piriform cortex), integrating olfactory inputs coming from the olfactory bulb, and the claustro amygdaloid pallial complex (Medina and Abellán, 2009). More precisely, the **lateral pallium generates the dorso-lateral**

claustrum and the baso-lateral amygdala, whereas the **ventro-medial claustrum, the ventral endopiriform nucleus and the lateral amygdala derive from the ventral pallium** (Puelles et al., 2000). The claustroamygdaloid complex has a non-laminar organization and integrates emotional inputs, with the claustrum as one of the brain domains suspected to be involved in consciousness by binding disparate events experienced at one point in time into a single percept (Crick and Koch, 2005), and the pallial part of the amygdaloid complex as a structure responsible for perception of pheromonal stimuli (olfactory amydala), or fear conditioning (frontotemporal amygdaloid system)(Maximino et al., 2013).

In terms of neuronal composition, the main characteristic of the pallium is the massive presence of projection neurons expressing glutamate as a neurotransmitter (Medina and Abellán, 2009).

1.1.2 The subpallium

The subpallium corresponds to the ventral telencephalon. It contains the basal ganglia, responsible for planning and controlling the voluntary movements (Kandel et al., 2000), and the preoptic area (PO) responsible for thermoregulation and receiving several inputs from thermoreceptors located throughout the body (Kandel et al., 2000). During development, it is subdivided into the lateral ganglionic eminence (LGE), the medial ganglionic eminence (MGE), the anterior entopeduncular area (AEP) and the preoptic area (PO) (Moreno et al., 2009).

The LGE generates the subpallial part of the amygdala involved in the control of the central autonomic autonomic nervous system, the "autonomic" amygdala complex, and the striatum, itself composed of two composed of two nuclei (the caudate nucleus and the putamen) (

Figure 1- CP/AuA). The MGE emerges during development ventrally to the LGE and generates the pallidum proper composed of the dorsal pallidum, also called the globus pallidus, and the ventral part of the pallidum (

Figure 1- Pal) (Moreno et al., 2009). Finally, the PO contains three major subdivisions, a novel commissural preoptic division (POC, at the base of the septum), the anterior preoptic area (POA) and the preoptic-hypothalamic border region (POH) (Moreno et al., 2009).

In terms of neuronal production, the subpallium is responsible, in addition to its own projection neurons, for generating GABAergic and cholinergic interneurons that integrate locally or migrate into several telencephalic regions, such as the cortex or the striatum (Marín and Rubenstein, 2001).

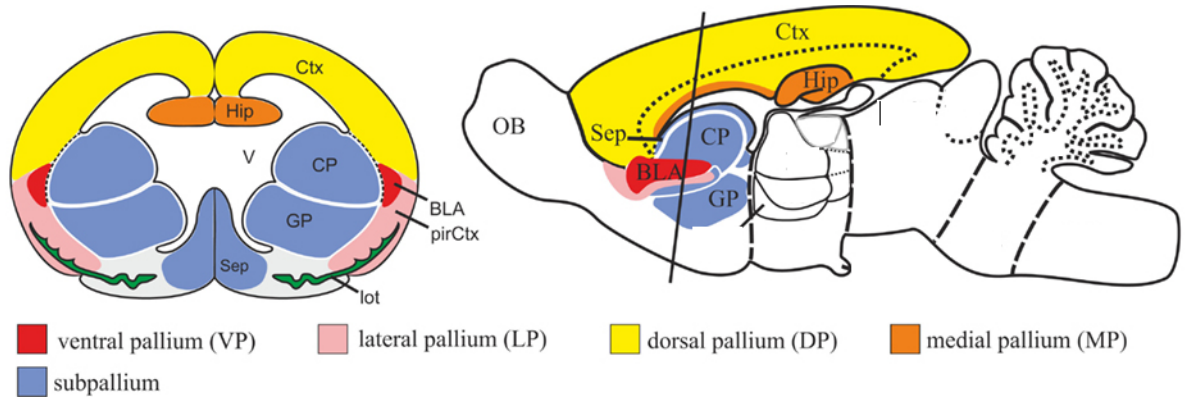


Figure 1: Schematic drawing illustrating the different pallial and subpallial subdivisions in the mammalian telencephalon.

The mouse pallium consists mainly of four divisions: a medial pallium (MP) corresponding to the mammalian hippocampus (Hip), a dorsal pallial division (DP) topologically corresponding to the mammalian isocortex (Ctx), and ventral (VP) and lateral (LP) pallial divisions corresponding to the mammalian pallial (basolateral) amygdala (BLA) and piriform cortex (pirCtx) respectively. Ventrally, the mouse subpallium is subdivided into the autonomic amygdala (AuA) and the caudate putamen (CP), the pallidum (Pal) composed of the pallidum proper and the glodus pallidus, and the pre-optic area that includes the septum (Sep). OB: olfactory bulb. Adapted from Mueller, 2012.

1.2 From a simple sheet of ectoderm to a patterned forebrain

Neural induction specifies embryonic ectodermal cells toward the more restricted fate of neuroectodermal cells. Today, in addition to the initially proposed 'default model' of neural induction, derived from experiments in frog conducted by Speeman (reviewed in De Robertis and Kuroda, 2004), a series of positive active processes have been discovered that also participate in neural induction. Overall, formation of the prospective forebrain includes two steps: ectodermal cells must acquire neural identity with an anterior character, and regional patterning must take place within the rostral neural tube.

In the following section, I will summarize current molecular knowledge on how anterior neural induction occurs. Next, I will address how the neural plate is further subdivided, thus generating the embryonic forebrain in the most anterior domain of the central nervous system.

1.2.1 Patterning of the anterior neural plate

According to the default model, ectodermal cells differenciate into neural tissue unless exposed to Bone Morphogenetic Proteins (BMPs) secreted from the ventral side of the gastrula. Thus, the first structure controlling anterior neural induction is the organizer itself,

which, at the onset of gastrulation, secretes BMPs signaling antagonists such as Noggin, Chordin and Follistatin, triggering neural plate induction from the dorsal ectoderm (Appel, 2000; Pera et al., 2014). It is now known that BMPs inhibition is not sufficient and that earlier signals act as neural inducing signals: the Fibroblast Growth Factors (FGF8) together with Insulin like Growth Factors (IGFs) pathways are required for neural induction before gastrulation, and act in favor of a caudalization of the tissue (Pera et al., 2014). These signals together are integrated at the level of the BMP signalling transducer Smad1, which is thus differentially phosphorylated (Eivers et al., 2009) and inhibition of its phosphorylation leads to the neural fate induction (Pera et al., 2003).

The Retinoïc Acid (RA) pathway, activated at the level of the organizer, also plays an important role in regulating the Chordin/BMP axis by reinforcing the posterior gradient of BMPs in the neural plate (Pera et al., 2014) (Figure 2, blue arrows). Whenever neural tissue is induced, it adopts by default an anterior fate, as revealed by *Otx2* expression. Thus, it seems that neural induction and acquisition of anterior identity are linked, and that later events posteriorize the neural plate. Rostral tissue must be protected from caudalizing factors to retain anterior characters. This is done by localized expression of antagonists of caudalizing factors and by morphogenetic movements to push the anterior neural plate away from the caudalizing factors. The organizer is initially the source of antagonists of caudalizing signals. But other structures also help protecting anterior tissues, such as the anterior visceral endoderm (AVE-mouse)/ Yolk Syncytial Layer (YSL-zebrafish), or the hypoblast (chick) (Andoniadou and Martinez-Barbera, 2013; Wilson and Houart, 2004). Later, the anterior axial mesendoderm (AME) participates in anterior fate protection. These structures underlie the rostral neural epithelium and protect the anterior neural plate from an active posteriorization by secreting BMP/TGFβ and Wnt/β-catenin antagonists such as Noggin, Chordin, Dickkopf-1 (Dkk1) and Cerberus (Cer1)(Andoniadou and Martinez-Barbera, 2013) (Figure 2, red arrows). The Nodal pathway has also a dual role in the neural induction. It acts itself as caudalizing factors but is also involved in the development of the mesendodermal tissue, participating in the "protection" of the anterior fate of the neural plate (Wilson and Houart, 2004).

To summarize, the neural plate is induced with anterior fate, then patterned via the establishement of two opposite gradients: one that inhibits posterior signals and one that promotes posterior. This leads to the first antero-posterior positional information delivered to the freshly induced neural cells within the tissue and will determine the future different brain regions (Figure 2).

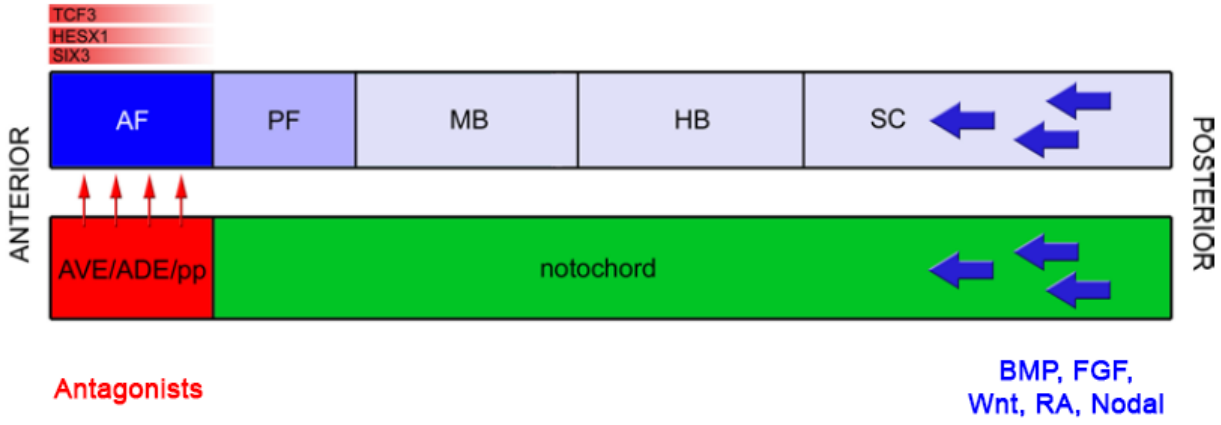


Figure 2: Signalling involved in the antero-posterior patterning of the neural plate

Signals exert a posteriorizing action on the neural plate (blue arrows). Antagonists of the respective pathways in the anterior forebrain, such as secreted Cerberus, Lefty1, Dkk1, Noggin, and Chordin (red arrows) are released by the underlying AVE, ADE, and prechordal plate. Within the prospective anterior forebrain, intrinsic factors such as Hesx1, Six3, and Tcf3, aid in regulating the competence of neural tissue to prevent ectopic posterior identity, possibly by preventing the expression of target genes of these pathways, hence maintaining anterior forebrain identity. AF: anterior forebrain, PF: posterior forebrain, MB: midbrain, HB: hindbrain, SC: spinal cord, AVE: anterior visceral endoderm, ADE: anterior definitive endoderm, pp: prechordal plate. Adapted from Andoniadou and Martinez-Barbera, 2013

1.2.2 Antero-posterior patterning of the forebrain

One consequence of this initial regionalization of the neural plate is the expression of different homeodomain transcription factors in broad but distinct domains along the AP axis. At boundaries between these TFs, expression of signaling molecules is turned on and “local organizers” are established. They modulate and refine patterning. This subdivides the anterior neural tube into segments: the forebrain, the midbrain, and the hindbrain.

The forebrain corresponds to the most anterior subdivision and derives from the anterior neural plate. Following its induction, the forebrain rapidly grows and is submitted to complex morphogenetic movements. By the end of somitogenesis, it comprises the dorsally positioned telencephalon and eyes, the ventrally positioned hypothalamus and the caudally located diencephalon subdivided into prethalamus, thalamus and pretectum (Wilson and Houart, 2004).

Local organizers have been identified that are involved in this arealization, and studies in zebrafish have been determinant in our understanding of this process. These studies have identified the **anterior neural border (ANB**) (or ANR -anterior neural ridge- in the mouse) located at the anterior-most border of the neural plate (Figure 3), as a local source of Tlc, a

member of the secreted Frizzled Related Proteins (sFRP) known to antagonize Wnt activity. Overexpression of *tlc* leads to an expansion of the telencephalic domain at the expense of the eye field (Houart et al., 2002) while overactivation of Wnt signaling, e.g. in *masterblind* (*mbl*) mutants which carry a mutation in the canonical Wnt pathway gene *axin1*, show a caudalized phenotype with an expansion of the diencephalon at the expense of rostral identity. This implies the presence of new sources of Wnt that need to be antagonized. Indeed, *Wnt8b* is expressed caudally to the telencephalon. This illustrates that different doses of Wnt activity trigger the specification of the different forebrain regions, with the telencephalic domain specified at very low levels of Wnt and the diencephalon induced at quite high levels of Wnt activity (Figure 3). The ability of the telencephalon to develop on low levels of Wnt seems to be conserved among several vertebrates such as frog, chick and mouse (Wilson and Houart, 2004). In terms of transcription factors, Hesx1, Six3 and the repressor Tcf3 participate in the inhibition of Wnt/ßcatenin targets in the anterior neural plate to prevent ectopic posterior identity (Andoniadou and Martinez-Barbera, 2013). All these factors are expressed within the anterior neural plate (Figure 2), and their respective mutants display impaired telencephalon, eye and hypothalamic development (Lagutin et al., 2003; Oliver et al., 1995).

In addition to Wnt signals, the Fgf pathway participates in refining forebrain patterning. *Fgf8* is expressed later by the ANB/ANR (Figure 3), but is rather required for cell survival and/or maintenance of the patterning and differentiation within the telencephalon, as telencephalic tissue is still induced in fish and mouse with compromised Fgf signaling. The cephalic neural crest cells, delaminating from the anterior dorsal border of the neural tube, also contribute to the maintenance of *Fgf8* expression in the ANB (Creuzet et al., 2006).

Interestingly, even though BMP inhibition is required to induce anterior neural fate, a recent study highlights that a certain level of BMP activity, going through the BMP2b receptor, is also necessary locally during zebrafish neural plate induction to maintain telencephalic fate and to subdivide it from the eye field (Bielen and Houart, 2012). This indicates that the BMP pathway also plays an instructive role in telencephalic specification, and that a balance of BMP and Wnt activity is important to properly specify the different forebrain structures (Figure 3).

The ***Zona Limitans Intrathalamica* (*ZLI*)** is established after the ANB and is a very important organizing center, necessary for diencephalic development by subdividing the pre-thalamus and the thalamus (Figure 3) (Wilson and Houart, 2004). It is the only region where *Sonic hedgehog* (*Shh*) expression is produced dorsally within the neural tube. A number of genes are expressed in the anterior neural plate with boundaries at the level of the prethalamus/thalamus boundary. For example, studies in chick suggested that the *Six3*/*Irx3* boundary prefigures the position of the *ZLI* (as mentioned, *Six3* is expressed in the most

anterior part of the forebrain, while *Irx3* is posterior) (Figure 3). Other genes such as *Hesx1*, *Fezf*, *Irx1* and *Otx* were more recently shown to influence ZLI positioning, and *Otx2* also initiates *Shh* expression. The expression of all of these genes is direcly or indirectly under the control of Wnt signaling, *Six3*/*Fezf* being repressed by Wnt while *Irx* posteriorly is activated by Wnt. The position of the *ZLI* also coincides with the underlying position of the prechordal plate/notochord boundary, suggesting a participation of the surrounding tissue in the establishment of the *ZLI* (Andoniadou and Martinez-Barbera, 2013).

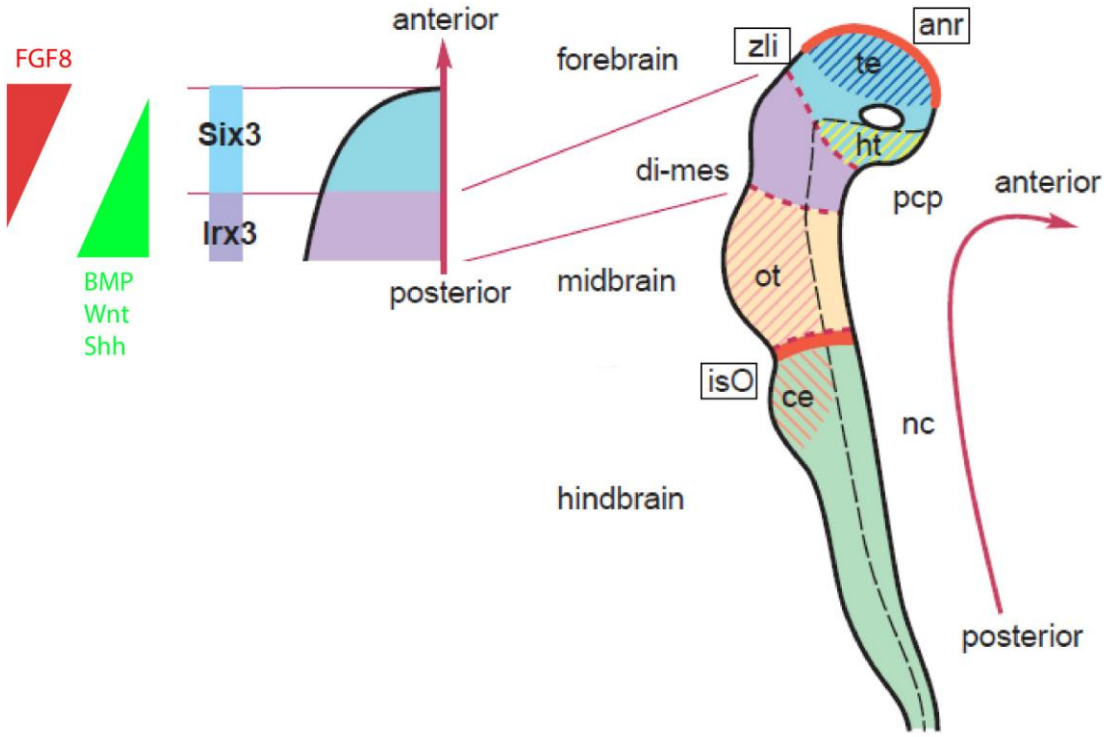


Figure 3: Model of early regionalization of the vertebrate anterior neural plate

Schematic representation of the patterning of the anterior neural plate of a chick embryo. The anterior neural plate is patterned and subdivided into different domains: the forebrain (eye, telencephalon, hypothalamus), the midbrain and the hindbrain. The forebrain and the midbrain are separated by the diencephalon-mesencephalon boundary (di-mes) and the midbrain/hindbrain boundary corresponds to the isthmic organizer (isO). Whithin the telencephalon, the pre-thalamus and the thalamus are separated by the Zona Limitans Intrathalamica (ZLI), which expresses Shh. The most anterior region corresponds to the anr (anterior neural ridge or Anterior Neural Border –ANB-), which expresses Wnt antagonists and later Fgf8. While Wnt and BMP ligands are expressed caudally to the telencephalon, their signaling is inhibited at the level of the telencephalon by several secreted antagonists and by the activity of transcription factors inhibiting Wnt targets (Six3). These signals also activate or inhibit the expression a set of transcription factors important for ZLI positioning (Six3/Irx3).

Longitudinal axes of the neural plate and tube are indicated by red arrows. anr, anterior neural ridge; ce, cerebellum; di-mes, dien-mesencephalic boundary; ht, hypothalamus; isO, isthmic organizer; nc, notochord; ot, optic tectum; pcp, prechordal plate; te, telencephalon; zli, zona limitans intrathalamica. Adapted from Kobayashi et al., 2002

1.2.3 Dorso-ventral patterning of the forebrain

The dorso-ventral patterning of the central nervous system relies on two opposite gradients generated from a source of Shh at the ventral floor plate and from a TGFβ/Wnt gradient at the dorsal midline. The role of these gradients and their interactions in Dorso-Ventral (D/V) specification has been extensively studied in spinal cord development. Indeed, together with RA signaling produced by lateral somites and activated in the spinal chord, these pathways generate very precise positional information along the D/V axis of the spinal cord and subdivide the progenitors of the neuroepithelium into domains that will generate particular types of neurons such as motor neurons ventrally and different interneuronal populations medially and dorsally (Bertrand and Dahmane, 2006).

In the forebrain, a similar role of Shh in the D/V patterning has been reported. Indeed, overexpression of *Shh* in the zebrafish embryo ventralizes the forebrain (Rohr et al., 2001), and ablation of *Shh* in the mouse leads to the loss of ventral telencephalic structures (Chiang et al., 1996). Similarly, *Fgf8* mutant mice show defects of ventral telencephalic specification (Storm et al., 2006), and analyses of *Fgfr* mutants indicate that Fgfs act downstream of *Shh* (Gutin et al., 2006). At least in the zebrafish, *Shh* expression is reinforced by Nodal activity in the telencephalon (Figure 4) (Rohr et al., 2001).

On the contrary, the transcriptional repressor *Gli3* is important for dorsal telencephalic structure specification. Indeed, mutant embryos for the *Gli3* factor display a loss of dorsal structures such as the choroid plexus and the hippocampus, and a lack of dorsal genes expression such as *Emx* genes (Grove et al., 1998; Theil et al., 1999). Moreover, analysis of *Gli3* mutant mice indicates that it would act upstream of *BMP* and *Wnt* expressed at the dorsal midline in the specification of the dorsal telencephalic structures (Kuschel et al., 2003) (Figure 4). As *Gli3* is antagonized by Shh, two opposite gradients of the repressor Gli3 dorsally and Shh ventrally are established within the developing forebrain and orchestrate the specification of both dorsal and ventral structures (Figure 4).

Interestingly, all these signals are integrated at the level of the transcription factor FoxG1. Shh and Fgf8 initiate *FoxG1* expression ventrally, and later *FoxG1* reinforces Fgf8 expression in the ventral telencephalon through a positive feed-back loop. *FoxG1* is necessary for subpallial specification as *FoxG1* mutant mice are incapable of turning on the ventral telencephalic program (Danesin et al., 2009). Dorsally, *FoxG1* expression is limited by *Gli3*, thus creating a ventro-dorsal gradient of *FoxG1* in the developing telencephalon that restricts *Wnt8b* transcription to the dorsal telencephalic roof plate (Danesin and Houart, 2012). In the cortex, *FoxG1* is then involved in promoting the production of cortical deep layer neurons (Hanashima et al., 2004).

As in the spinal cord, RA signalling is also involved in D/V telencephalic patterning. It refines the information delivered by the different D/V gradients to generate three different

compartments within forebrain structures: the dorsal compartment, the intermediate compartment, and the ventral compartment (Figure 4) (Bertrand and Dahmane, 2006). The sources of RA could correspond to the head ectoderm and the lateral olfactory placodes that both express enzymes for RA production (Blentic et al., 2003; Paschaki et al., 2013). Studies in the chick embryo indicate that ventral Shh and Fgf8 expression are partially dependent on RA activity, and Fgf8 blocks RA action in the most ventral telencephalic cells (Marklund et al., 2004; Schneider et al., 2001). Moreover, embryos treated with an RA antagonist display a loss of *Meis2* expression, a marker of intermediate striatal progenitors, while dorsal *Pax6/Emx1* and ventral *nkx2.1* expression are still present, indicating that RA is necessary for the specification of the intermediate telencephalic character (Figure 4) (Marklund et al., 2004).

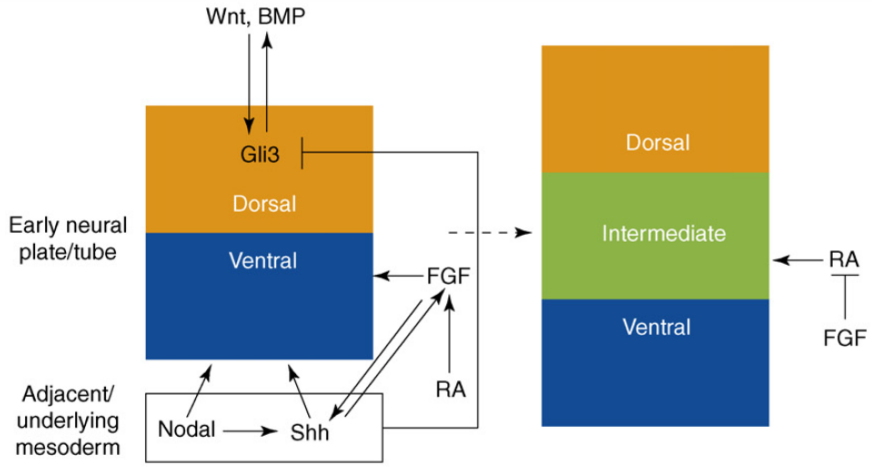


Figure 4: Selected interactions between signaling pathways at early (left; E8.5 to E9) and later (right) forebrain development in the mouse embryo.

The localization and ranges of signaling molecules change with time and are affected by tissue growth. Arrows represent positive influences and T bars negative ones. These influences can occur at many levels, including transcriptional, post-transcriptional and effects on cells that then express other signaling molecules (Bertrand and Dahmane, 2006).

As growth proceeds, the distance between the different “local” organizing centers responsible for the first wave of patterning of the neural plate increases, and new organizing centers emerge within the developing telencephalon and especially in the pallium: the pallial/subpallial boundary (PSB), the postero-medially cortical Hem (Hem) and the antero-medially commissural plate (Co-P). They participate to the refinement of dorsal telencephalic patterning and are thus involved in the development of the different pallial structures, and particularly in cortex arealization.

1.3 Regionalization of the telencephalon – lessons from mouse

Following their specification and early regionalization via the different gradients influencing neural plate patterning, the pallium and subpallium will next be submitted to proliferation and morphological changes to shape the adult telencephalon. This is achieved, at least in part, via a "homeodomain code". This section will deal with the role of this code in shaping telencephalic complexity from a simple tube structure.

1.3.1 Telencephalic subdivisions: A molecular code integrating D/V and A/P patterning

Patterning of the embryonic telencephalon leads to the generation of different subdivisions with cells acquiring a "dorsal" or a "ventral" identity, and a population of cells in which an "intermediate status" is imprinted. This patterning is regulated by a "molecular code" based on several transcription factors and particularly on bHLH and homeobox factors (Figure 5), which are regulated by the pathways involved in the D/V and A/P patterning of the forebrain.

In the ventral side, the main genes involved in regionalization are *Nkx2.1/2.2*, *Gsx2 (previously Gsh2)* and *Dlx1/2/5* genes (Figure 5). Their expression are partially controlled by the Shh and Fgf8 pathways and their combination defines the different zones of the ventral telencephalon, the subpallium. For instance, *Gsx2* is expressed in both the lateral and medial ganglionic eminence (LGE/MGE), and the MGE is characterized by strong *Dlx1/2/5* and *Nkx2.1* expression and weak *Nkx2.2* expression (Moreno et al., 2009; Wilson and Rubenstein, 2000). Moreover, the bHLH factor Mash1 defines the ventral telencephalon and has been shown to be involved in the subpallial production of GABAergic interneurons (Casarosa et al., 1999).

As we have seen previously, the RA and FGF pathways specify the intermediate compartement. This level of the telencephalon corresponds to the area where some of these genes, specifying the dorsal and ventral subdomains, meet each other and thus define the pallial/subpallial boundary (PSB). This intermediate compartment is characterized by *Meis2* expression delimited dorsally by *Emx1* expression, dedicated only to the dorsal telencephalon, and ventrally by *Nkx2.1* specific of ventral structures (Marklund et al., 2004). This intermediate domain is also subdivided by the boundary between the pallial marker *Pax6* and the subpallial marker *Gsx2*, namely the pallial/subpallial boundary (PSB) (Cocas et al., 2011; Marklund et al., 2004). In mammals, lineage tracing experiments of *Pax6*- and *Gsx2*-positive cells have shown that the PSB is progressively refined during development and creates a strict boundary at E15.5 (Cocas et al., 2011). The ventral pallial part of the intermediate compartement is characterized by *Dbx1* expression (Medina et al., 2004), and *Pax6* participates in its activation as *Pax6* mutant mice display a severe reduction of *Dbx1*

expression (Cocas et al., 2011). This intermediate telencephalic compartment comprising the ventral pallium and the LGE will generate the striatum, the olfactory bulbs and part of the amygdala (Marklund et al., 2004).

Dorsally, the patterning of the cortical plate is achieved by successive steps in which different pallial organizers are involved (Figure 6A). The cortical Hem and the commissural plate, which express respectively Wnts and BMPs, and FGFs (FGF8 and FGF17) ligands, together with the PSB (see section 1.3.1), expressing the Wnt antagonists SFRP2 and several EGF factors, create patterning gradients of transcription factors involved in the arealization of the cortex. At stages of development where no morphological landmarks can predict the boundary of the future cortical areas, *Pax6* and *Emx1/2* homeobox genes pattern the rostro-caudal axis of the pallium, based on two opposite gradients: *Pax6* is strongly expressed in the rostro-latero-ventral pallium while *Emx1/2* expression is located in the caudo-medial-dorsal pallium (Figure 6B) (Wilson and Rubenstein, 2000). In addition to these two molecules, a postero-lateral gradient of Coup-TF1 and an antero-medial gradient of Sp8 have been reported, and are highly involved in the arealization of the cortex. Absence of one of these four transcription factors generate changes in the repartition of the different cortical areas (Figure 6B) (O'Leary and Sahara, 2008). *Pax6, Emx2, Coup-TF1and Sp8* are also able to regulate each other's expression and thus participate in the refinement of the gradients (Figure 6C). Their opposing activities determine positional information and imprint the arealization in the developing cortex (Borello and Pierani, 2010). In parallel to the subdivision of the cortical areas, markers of progenitor commitment appears such as *T-box brain1* and *2* (*Tbr1* and *Tbr2*), activated downstream of Pax6. They highlight the production of glutamatergic neurons in the cortex (Englund et al., 2005; Puelles et al., 2000).

This information are relevant for the present work as I studied the regionalization of the early telencephalon and make links between these embryonic regions and the adult telencephalic territories, by combining lineage tracing of particular embryonic telencephalic domains with expression of either transcription factors such as Tbr1 or Gsh2 but also components of signaling pathways such as Fgf8 or Wnt3a.

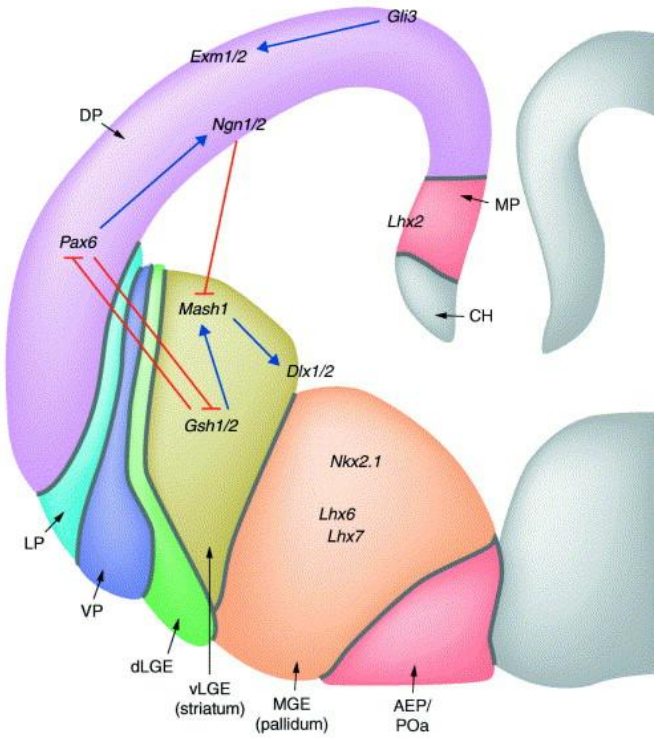


Figure 5 : Genetic interactions underlying the dorso-ventral regionalization of the mammalian telencephalon.

Schematic coronal section through the telencephalic vesicles at E12.5 showing dorsal and ventral subdomains, as defined by their unique genes expression pattern and highlighting the different interactions between the transcription factors that participate in telencephalic regionalization. Arrows denote positive interations; T-bars denote inhibitory control. (Schuurmans and Guillemot, 2002)

1.3.2 Cortical patterning via the migration of signaling cells.

As already mentioned, the establishment of these gradients triggers expression of a large list of secondary transcription factors, specifying the anterior motor area, the medial somatosensory area, the postero-lateral auditive area and the postero-medial visual area (Rakic et al., 2009). These gradients initially emerge from organizing pallial centers – ie. the cortical Hem (Hem), the commissural septum (Co-P) and the pallial/subpallial boundary (PSB) (Figure 6A). Nevertheless, it is worth noting that pallial centers are discrete zones compared to the large cortical plate. Moreover, their sizes are stable already at E10.5, a stage at which the pallial gradients can still be modulated, indicating that another level of regulation is involved in the formation of these gradients (Borello and Pierani, 2010; O'Leary and Sahara, 2008; Shimogori et al., 2004a). The Cajal and Retzius (CR) cells, so far only found in the mammalian cortex, are among the first neurons produced in the developing pallium. Three distinct populations arise from E10.5-E11.5 respectively from the organizing centers of the pallium (Hem, PSB, Co-P) (Bielle et al., 2005). They migrate and establish "zones" at the surface of the cortical primordium. Their role is to propagate within the cortical plate the signals of the organizers they come from, thus to participate in the establishment of the different patterning gradients of transcription factors (Figure 6B). Indeed, changing the dynamics of the distribution of CR cells, via genetic manipulations, influences the early gradient of transcription factors, thus triggering changes in the position and size of the different cortical areas (Griveau et al., 2010).

In addition to their role in arealization, Cajal-retzius cells positioned at the surface of the cortical plate in the marginal zone regulate, via their production of the Reelin protein, the radial migration of the cortical newborn neurons coming from the germinal zones, and thus participate in the generation of the laminar organization of the cortex (Tissir and Goffinet, 2003).

Finally, several other extrinsic telencephalic factors have been shown to influence cortical development such as the adjacent tissues (meninges), the ingrowing vasculature or the thalamocotical axonal projections (Borello and Pierani, 2010).

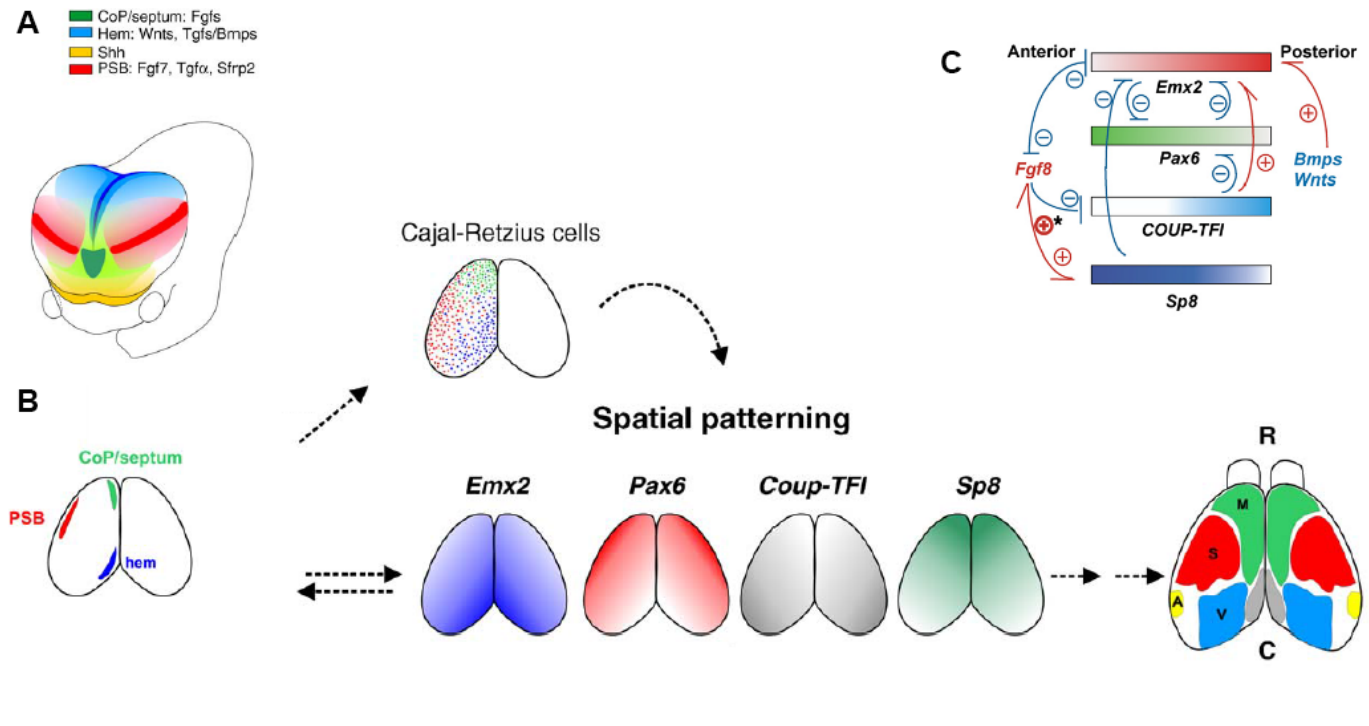


Figure 6: Molecular mechanisms for the antero-posterior patterning of the cerebral cortex: intrinsic and extrinsic cues

(A-B) Signaling molecules expressed at patterning centers (A) regulate the graded expression of specific transcription factors (Emx2, Pax6, Coup-TFI, and Sp8). These factors are involved in the early regionalization of the cortical primordium and, thus, in the position, size and identity of functionally distinct cortical areas in the postnatal animal. Dashed arrows indicate that direct transcriptional regulation has not been demonstrated in most cases. (B). The production of Cajal–Retzius subtypes by the different patterning centers modulate early cortical patterning by transporting signaling molecules over a long distance. CoP: commissural plate; PSB: pallial–subpallial boundary or anti-hem; hem: cortical hem; RA: retinoic acid; R: rostral; C: caudal; M: frontal/motor area; S: somatosensory area; A: auditory area; and V: visual area.

(C) In the anterior signaling center, Fgf8 establishes the low anterior-graded expression of the Emx2 and COUP-TFI transcription factors by repression, and promotes the high anterior gradient of Sp8 expression. Fgf8 expression is also regulated positively by direct transcriptional activation by Sp8, and indirectly by Emx2, which represses the ability of Sp8 to directly induce Fgf8. The asterisk marking the activation of Fgf8 by Sp8 indicates the only interaction that has been shown to be due to direct binding and transcriptional activation (Sahara et al., 2007). Putative posterior signaling molecules Bmps and Wnts, expressed in the cortical hem, positively regulate the high caudal gradient of Emx2 expression. Genetic interactions between TFs also participate in the establishment of their graded expression. +: positive interaction; - , negative interaction.

Adapted from (Borello and Pierani, 2010; O'Leary and Sahara, 2008).

1.3.3 Cortical progenitors

The production of cortical neurons is performed via two temporarily distinct progenitor zones: the embryonic ventricular zone (VZ), and the subventricular zone (SVZ). The VZ is composed of radial glial cells (RGCs) expressing glial markers such BLBP or GFAP, and displaying interkinetic nuclear migration, a process defined as an apico-basal movement of the nucleus within the cytoplasm of a progenitor and that allows it to progress into the cell cycle (see section 2.1.1). RGCs compose the cell layer directly lining the telencephalic ventricle during development and are connected with both the ventricular and pial surfaces (Götz and Huttner, 2005). They correspond to neural progenitors in almost all the regions of the central nervous system (Anthony et al., 2004; Malatesta et al., 2003), and are also necessary for positioning the different neuronal layers. During corticogenesis, they generate first the Cajal-Retzius cells located in the preplate (PP) and then in the marginal zone (MZ). Then, RGCs generate the deep layer neurons of the subplate, and the projection neurons of layer V and layer VI (Aboitiz and Zamorano, 2013). The SVZ is composed of intermediate progenitors cells (IPCs) that emerge from ventricular RGCs, setting up a new progenitor region immediately “above” the RG layer. The latter generates layers II, III and IV of the cortex (Figure 7). Contrary to RGCs, the SVZ IPs do not display interkinetic nuclear migration, nor connect with the ventricular and pial surfaces, but instead they have multipolar processes (Kriegstein and Alvarez-Buylla, 2009). Through their symmetrical divisions, they contribute to an amplification of the number of neurons produced during development. The existence of a third germinal zone during corticogenesis was recently recognized, the outer-subventricular zone (OSVZ) progenitors or outer radial glia (ORGCs). Initially described in human and ferret but also present in rodent, these progenitors maintain only a basal attachement at M-phase, divide in the OSVZ and are supposed to derive from bipolar RGCs (Fietz and Huttner, 2011). The number of times the subventricular and outer-subventricular progenitors divide varies in different cortical regions and different species, such as in primates in which the OSVZ contributes to an enormous cortical expansion (Fish et al., 2008).

Finally, in addition to the neurons produced from these cortical germinal zones, the cortex is composed of GABAergic interneurons that are generated in the subpallial VZ and migrate tangentially to integrate into the cortical network (Marín and Rubenstein, 2001). The subpallial-pallial tangential migration plays a critical role in the establishment of the thalamo-cortical connectivity with a role in the guidance of the thalamic neurons axons (Molnár et al., 2012).

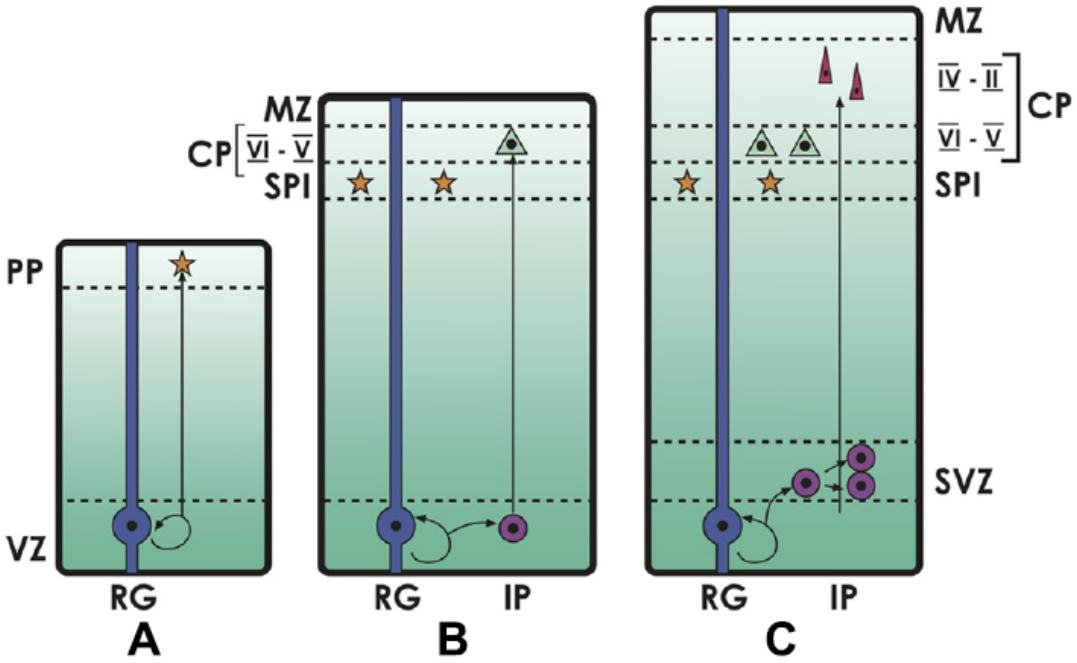


Figure 7: Neocortical development in rodents

The deep ventricular zone (VZ) and the subventricular zone (SVZ) are the compartments where cell proliferation takes place. (A) In early cortical development, primary neural progenitors or radial glia (RG) in the VZ divide and give rise to early neurons that migrate to the preplate (PP), and then make up the embryonic subplate (SPI).
(B,C) Later in development, RG generate intermediate progenitors (IP), that keep dividing and producing neurons into the emerging cortical plate (CP, future layers VI–II of the neocortex), in an inside-out gradient where deep layers (VI–V) are formed first and mostly derive from progenitors in the VZ, and superficial layers (IV–II) are formed later, deriving from progenitors in the SVZ. The most superficial layer (layer I) is the remnant of the embryonic marginal zone (MZ), in which Reelin-producing Cajal-Retzius neurons are located. (Aboitiz and Zamorano, 2013)

1.3.4 Hippocampus development

The hippocampus develops at a caudal level between the isocortex and the cortical Hem organizing center. Cortical Hem signals have been shown to be essential for early hippocampal development, however, how the Hem is positioned in still under debate and several models have been proposed (Subramanian and Tole, 2009). During early cortical development, first *Wnt3a* and later *Wnt5a* and *Wnt2b* are expressed in the Hem (Figure 8). Mutation in *Wnt3a* or its target gene *Lef1* alter the development of the hippocampus, indicating a crucial role of this pathway in hippocampal formation (Galceran et al., 2000; Zhou et al., 2004). Recently, BMP, weakly expressed in the cortical Hem structure but strongly expressed in the adjacent choroid plexus, has been shown to regulate hippocampus

formation via maintaining Wnt signaling in the Hem (Figure 8) (Caronia et al., 2010). In addition to this, the main transcription factor family regulating dentate gyrus development is the LIM family. Indeed, *Lhx5* is expressed in the cortical Hem (Figure 9) and its disruption leads to a shortening of the cortical Hem at the expense of the medial pallium and to defects in hippocampus formation (Zhao et al., 1999). On the contrary, *Lhx2* is expressed in the medial pallium and thus delimits the cortex-Hem boundary (Figure 8 and Figure 9A). Similarly, *FoxG1* is excluded from the cortical hem, and mutations of *FoxG1* or *Lhx2* lead to an expansion of the cortical Hem, indicating that they are involved in delimiting the proper size of the structure (Figure 9 Figure 8 and Figure 9A) (Li and Pleasure, 2007). In addition, *Fgf8* regulates midline fate in the telencephalon by inducing expression of transcription factors such Lhx5 specifically in the hem, and repressing *Lhx2* (Okada et al., 2008).

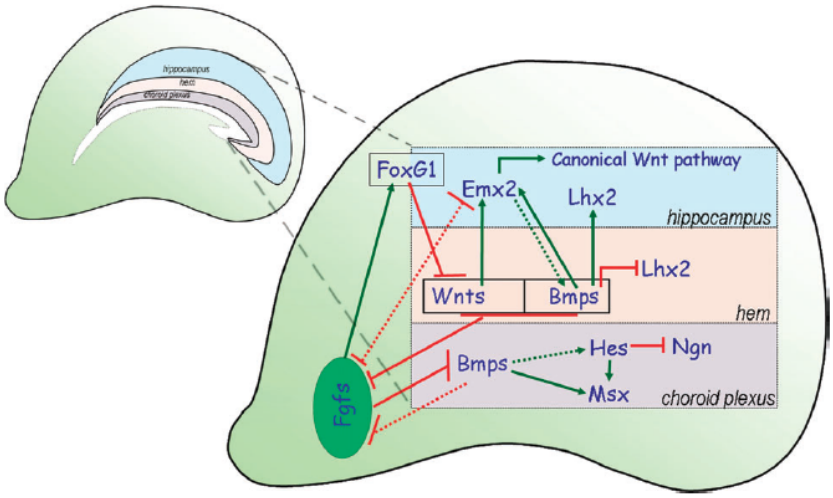


Figure 8: A schematic of the medial telencephalon focusing on known molecular interactions that pattern the hem and choroid plexus

Solid lines indicate a direct interaction; dashed lines indicate an indirect or an inferred interaction. Green arrows indicate activation, whereas red bars indicate repression. (Subramanian and Tole, 2009)

Already at embryonic stage, within the hippocampus presumptive neuroepithelium, two zones are distinguishable: the dorsal ammonic neuroepithelium (AN), which will generate the pyramidal neurons of the CA1/2/3 fields, and the ventral dentate neuroepithelium (DNe), at the origin of the dentate gyrus and abuted by the cortical Hem (Rolando and Taylor, 2014; Yu et al., 2014). The first step in the formation of the hippocampus is, at around E15, the relocalization of dentate gyrus precursors via their tangential migration toward the limit

between the meninges and the fimbria, a ventral prominent band of efferent axons, through a “subpial” migratory stream (Figure 9B) (Rolando and Taylor, 2014; Yu et al., 2014). This step may be regulated by chemo-attractant signals and the Integrin pathway (Li and Pleasure, 2007). Interestingly, recent lineage tracing experiments using the Wnt signaling receptor Frizzled10 indicate that Cajal-Retzius cells colonize the hippocampal marginal zone and migrate along the fimbrial scaffold during hippocampal formation (Gu et al., 2011). Some of the CR progeny persist in the post-natal hippocampus (Gu et al., 2011), but their precise function remains to be determined. This migration event leads to the formation of the subpial zone (SPZ) (Figure 9C), and, at early postnatal stage (P5), progenitors residing in the SPZ undergo a redistribution to generate the neurogenic zone of the adult hippocampus, the sub-granular zone (see section 2.2.2) (Figure 9D) (Li and Pleasure, 2007; Rolando and Taylor, 2014; Yu et al., 2014).

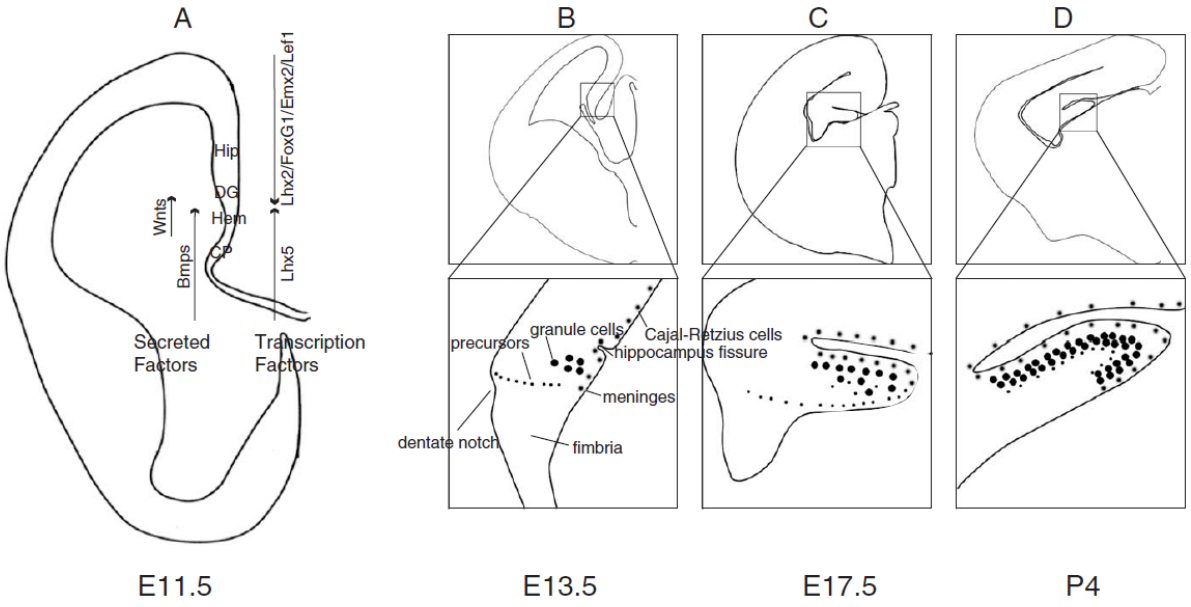


Figure 9: Schematic diagram showing the anatomical events and some of the genes involved in dentate morphogenesis.

(A) Schematic representation of a cross section of a mouse telencephalon at E11.5 with the cortical hem adjacent to the dentate neuroepithelium. The expression domains for Wnts and BMPs are shown with arrows to the left of the neuroepithelium. The expression domains of Foxg1, Lhx2, Emx2, and Lef1 are shown to the right of the neuroepithelium and their extent is indicated with arrows. (B-D) Zoom on the developing hippocampus showing the initial migration of precursors from the dentate notch to the forming dentate gyrus and the emergence of the Cajal-retzius cells from the cortical hem at E 13.5 (B), the mix of the precursor cells and granule cells and the continued dentate precursor migration along the subpial migratory course at E17.5 (C), and finally, the radial reorganization of the dentate by condensation of the granule cell layers and positioning of precursors in the subgranular zone (D). DG: dentate gyrus, Hem: cortical hem, Hip: hippocampus.(Li and Pleasure, 2007)

In parallel to the migration of the precursors, the pyramidal neurons of the different CA layers are generated from the ammonic neuroepithelium. Little information is avalaible on the formation of CA fields. The layer specific-pyramidal cells are distinguishable by factors that persist from embryonic to adult stages, such as the *glutamate receptor KA1* gene expressed only in CA3 cells, or the POU-domain gene *SCIP* expressed in the CA1 layer. These two genes start to be expressed respectively at each pole of the developing hippocampus at around E15, and their expression progress toward each other to fuse in the CA2 layer, determined between CA3 and CA1 (Figure 10). The expression of these two field markers is cell-autonomous as they do no need the cortical Hem Wnt3a signal to be expressed, but the signals triggering their expression are still unknown (Khalaf-Nazzal and Francis, 2013).

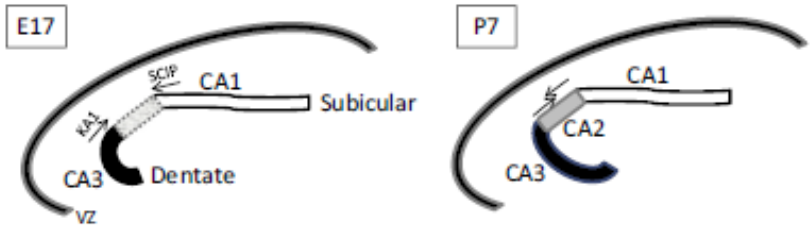


Figure 10: Embryonic and mature hippocampal pyramidal cell layers are identified by the expression of field specific markers KA1 and SCIP

The hippocampus is schematized at E17 and at P7. SCIP, for example, marks the CA1 field depicted in white, is expressed in the embryonic hippocampus as early as E15.5, and persists into adulthood. KA1, a CA3 specific marker, is expressed in the embryonic CA3 region depicted in black, and persists in the adult. During embryogenesis, expression of these respective markers starts at the subicular and dentate poles to finally join in the CA2 region (shown in gray in the P7 schema). From Khalaf-Nazzal and Francis, 2013

1.4 The zebrafish pallium: What is what?

The term “homology” between two structures in different organisms is used when they derive from the same territory present in a common ancestor (Cracraft, 2005). As we never have access to this common ancestor, two different strategies can be applied to determine homologies. The first strategy, to use with great caution, is to study features such as the topological environment of the structure of interest and its relationship with the surrounding organs or territories. In the case of the brain, neuroanatomists use arguments from the cytoarchitecture (the cell composition of the structure), the neurochemistry (neurotransmitters/ neuropeptides), and the hodology (the connectivity) of the territory to

propose whether two putatively homologous structures share some common features. The second strategy consists in studying the developmental history of the structure of interest, and compares this origin between species. The common embryonic origin of two territories support that a common ancestral structure would exist during evolution (Mueller and Wullimann, 2009). In the literature, until now, strategies to elucidate the homologous territories of the different pallial areas in the zebrafish compared to mammals were based on adult patterns of neuronal organization or on gene expression during development (Braford, 2009). In the following section, we will try to expose the different theories of homologous pallial domains between zebrafish and mouse, keeping in mind that this question is still under debate in the neuroanatomy field and that information on the developmental origin of the different adult brain structure in zebrafish are still largely missing.

1.4.1 Subdivisions of the zebrafish pallium and the concept of eversion

As discussed already, the pallium in all tetrapods is divided into 4 different regions (ventral, lateral, dorsal and medial pallia). This is based on both analyses of connectivity and expression patterns of regionalization genes, such as *Emx1/2* or *Pax6* (Medina and Abellán, 2009). Areas in different species were shown to be homologous to the mammalian pallial domains, such as the hyperpallium in birds homologous as a field to the isocortex, or the dorsal ventricular ridge (DVR) of the sauropsids homologous to the mammalian claustroamygdaloid complex (Medina and Abellán, 2009).

The zebrafish is a teleostean fish, characterized by its everted telencephalon, and belongs to the actinopterigian class. This particular morphology of the teleost pallium has made it especially difficult to compare with other vertebrates. Compared to mammals, chick, or lizard, in which the telencephalic roof invaginates during the first steps of forebrain development (inversion process) (Figure 11A), the zebrafish pallium is thought to develop following an outward bending of its lateral wall, leading to stretching the roof plate that generates a thin epithelium covering the ventricle, called the tela choroida (Figure 11B). It may result in a medio-lateral reversal of the four tetrapod pallial areas. Several theories concerning the eversion process and the homologies of the different pallial territories have been proposed (see section 1.4.2).

Another important difference between the pallium of mammals and non-mammals is related to lamination. Indeed, compared to mammals in which most of the pallial structures are laminated such as the isocortex and the hippocampus, the non-mammalian pallium, such as in amphibians or birds, displays a non-laminar but rather nuclear organization (Braford, 2009; Medina and Abellán, 2009). It is interesting to note that some structures in the mouse pallium

possess a non-laminar organization as well, such as the claustral complex or the basal complex of the amygdala (Medina and Abellán, 2009).

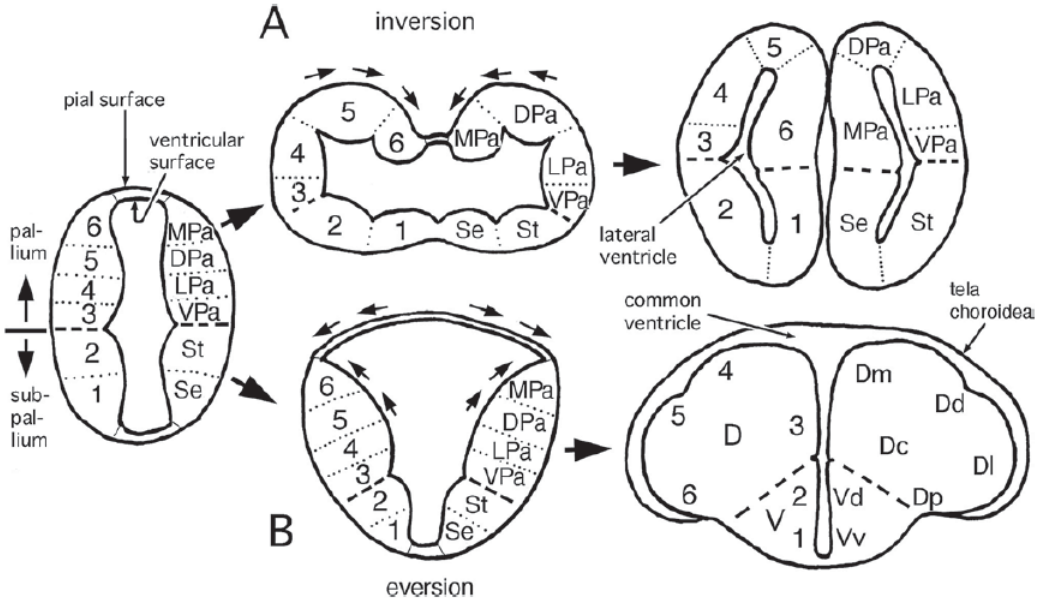


Figure 11: Schematic drawings showing telencephalic development through inversion in non-actinopterygian vertebrates (A: mammals) and through eversion in actinopterygians (B: teleost)

MPa: medial pallium, DPa: dorsal pallium, LPa: lateral palliul, VPa: ventral pallium, St: striatum, Se: septum, D: area dorsalis telencephali, Dl: area dorsalis telencephali pars lateralis, Dm: area dorsalis telencephali pars medialis, Dp: area dorsalis telencephali pars posterior. Dd: area dorsalis telencephali pars dorsalis (Yamamoto et al., 2007)

1.4.2 Zebrafish pallial regionalization and homology

The number of subdivisions present in the zebrafish pallium is still under debate. Most neuroanatomists consider that four different periventricular regions are present in the zebrafish pallium: medial pallium (Dm), dorsal pallium (Dd), lateral pallium (Dl) and posterior pallium (Dp), along with a central pallium (Dc) (Figure 11B) (Braford, 2009).

- The Dm domain: its lesion in goldfish impairs avoidance learning, suggesting that Dm is functionally homologous to the mammalian amygdala, involved in fear conditioning (Portavella et al., 2004). However, it is worth mentioning that the lesions performed in these experiments are quite large and do not allow a precise positioning of the pallium region responsible for a particular behavior. In addition to these functional experiments, connections ressembling thalamo-amygdalar inputs terminate into Dm in actinopterigians, and thus would make Dm a good candidate for a ventral pallium homolog (comprising part of the amygdala

in mammals). Nevertheless, there is no consensus concerning the hodology of this domain, and some neuroanatomists have identified these connections as thamalo-cortical rather than thalamo-amygdalar, thus considering Dm as part of the dorsal pallium (comprising the isocortex in mammals) (Braford, 2009; Yamamoto et al., 2007).

- **The Dd domain**: very little information exists concerning the role of the Dd domain. It could be in the territory homologous to the mammalian dorsal pallium, but this is mainly based on its position. Nevertheless, its proper function is still not clearly determined in teleosts, and depending on authors, the dorsal pallium would also comprise other parts of Dm and/or part of Dl (Braford, 2009).
- **The Dl domain:** lesions in the lateral pallium of goldfish result in the impaired encoding of the geometric information of environmental space (whereas medial pallium ablations do not give such a phenotype). This type of information is encoded by the mammalian and avian hippocampus (Vargas et al., 2006). Moreover, hodology reveals that Dl receives typical connections specific of the mouse medial pallium (Northcutt, 2006), leading to the conclusion that Dl hosts territories homologous to the medial pallial area of mammals.
- **The Dp domain**: it is the most problematic domain when we address the question of eversion. Indeed, in the simple eversion concept, it would correspond due to its position to the medial pallium (thus containing the hippocampus). Even though this hypothesis is supported by some neuroatatomists such as Nieuwenhuys (Nieuwenhuys, 2009), some studies in teleosts based on the morphology and hodology of this domain suggest that it would rather correspond to the mammalian lateral pallium, containing the piriform (olfactory) cortex in mammals (Braford, 2009).

The different theories of homology

As the simple eversion seems to be an incomplete model, neuroanatomists have elaborated theories to try and explain the homologies of the pallial domains of the zebrafish with mammalian subdivisions. Depending on the neuroanatomist, the criteria used diverge with some of them considering developmental criteria as the most appropriate to elaborate homologies, and others prefering the study of the adult structure itself with morphological and hodological features. I have tried to summarize the main theories that exist concerning teleost and mammalian brain homologies.

The first theory, postulated by Wullimann and Mueller in 2004, is mainly based on developmental gene expression (Figure 12A). In this model, Dp derives from the medial domain and its lateral location is explained by a migration from a medial to a lateral position. Caudally, Dp is adjacent to Dm, supporting the idea of an initial medial ventricular position of Dp between Dm and Dd. Following this idea, Dp would thus not be involved in the eversion process and this theory is thus called the “partial eversion”model (Wullimann and Mueller,

2004). Then, concerning the rest of the pallial area, the ventral pallium would correspond only to the ventral part of Dm, the lateral pallium would be the Dp domain, the dorsal pallium is formed from the dorsal part of Dm, the entire Dd and Dc domains and the dorsal part of Dl, and finally, the medial pallium would be only the ventral part of Dl. Recently, Mueller and collegues have proposed a revised view of this theory, based on gene expression and BrdU analysis (Figure 12B), in which they propose that the Dd domain is actually not a separate anatomical domain and is anteriorly the ventricular part of Dc and, posteriorly, the most medial part of Dl. Dm and Dp were still considered respectively as the ventral pallium and the "displaced" lateral pallium (Mueller et al., 2011a).

The second model, called "caudolateral eversion and displacement", has been developed by Yamamoto et al (Figure 12C). In this version, based on hodology criteria, Dm is not considered as the homologous part of the ventral pallium, but would be the nucleus taenia, a small region located ventro-laterally in the posterior pallium, along the pallium/subpallium boundary; however, together with the Dd/Dc domains and the dorsal part of Dl (Dlp), Dm forms the dorsal pallium. The lateral pallium, as for the previous model, is homologous to the Dp domain. Finally, the medial pallium is restricted only to the ventral part of Dl (Dlv)(Yamamoto et al., 2007).

Finally, the last hypothesis for eversion, explained by Nieuwenhuys in 2009 (Figure 12D), considers Dp as the ventral part of the Dl domain based on the topology and its attachment to the tela-choroida, the cover of the ventricle. In this model, the olfactory tracts that innervate massively the Dp domain are considered as newly derived (apomorphic) in actinopterigians, and thus Dp would be homologous to the medial pallium, and Dd and Dm respectively to the dorsal and the lateral pallium (Nieuwenhuys, 2009).

This question of homology of the pallial territories between mouse and fish is still not solved and information concerning the developmental origin of the different domains is missing to further understand how the eversion process imprints the structure of the everted pallium and to discriminate between these different models. Some of the results I obtained during my thesis bring up new information allowing to go further on this question, and especially on the embryonic origin of the Dp/Dl domains.

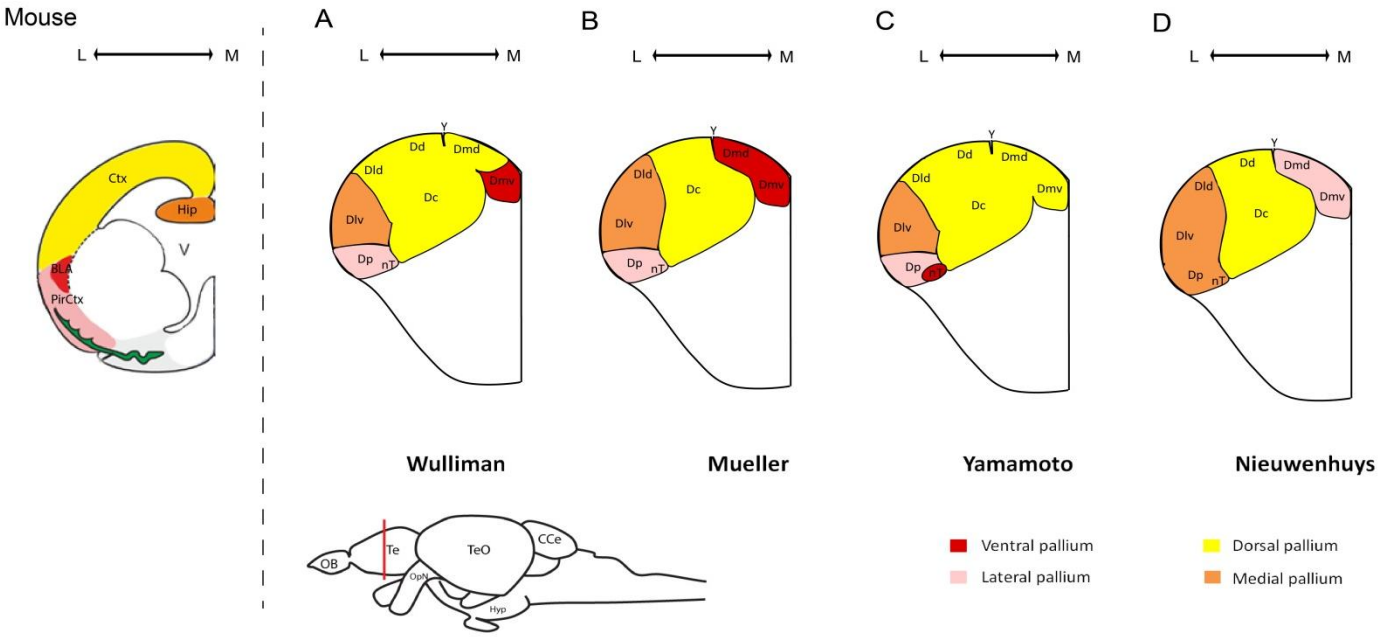


Figure 12: Summary of the different theories about homologies between the zebrafish and mammalian pallial domains

Right hemisphere of an adult zebrafish telencephalon at the level indicated in red in the lateral view of the entire adult zebrafish telencephalon in which are summarized the different homologies models (A-D) compared with the mammalian regionalization (left). Hip: Hippocampus, Ctx: isocortex, BLA: Baso-lateral amygdala, PirCtx: piriform cortex, Dp: area dorsalis telencephali pars posterior, Dlv: ventral part of the area dorsalis telencephali pars lateralis, Dld: dorsal part of the area dorsalis telencephali pars lateralis, Dd: area dorsalis telencephali pars dorsalis, Dmv: ventral part of the area dorsalis telencephali pars medialis, Dmd: dorsal part of the area dorsalis telencephali pars medialis,Dc: area dorsalis telencephali pars centralis. M:medial; L:lateral. Adapted from

During my thesis, I studied the origin of pallial stem cells but it also gave an information on pallium development and regionalization. By comparing it with what we know already about the formation of the mammalian pallial domains, it enabled us to determine the potential common mecanisms of telencephalon development and the homologies of the different pallial territories between the mouse and zebrafish.

1.4.3 How is eversion initiated?

In terms of development, Folgueira and al, based on clonal analysis and 3D reconstructions of embryonic zebrafish brains, recently brought up new information on how the eversion process could be initiated in the zebrafish telencephalon during embryonic development. They showed that two steps are necessary to generate an everted telencephalon. First, the formation of a deep ventricular recess between the telencephalon and the diencephalon, the anterior intraencephalic sulcus (AIS), creates a posterior ventricular wall to the dorsal domain of the telencephalon, thus displacing laterally the most postero-dorsal telencephalic regions

(Figure 13A). Second, a repositioning dorsally of the posterior ventricular wall and an expansion up to larval stage of the telencephalon along the A/P axis enable the formation of the pallium and the tela choroida (Figure 13B-C). This model is thus more complex than just a simple eversion that rolls out the dorsal telencephalon to a lateral position equally at rostral and caudal levels (Folgueira et al., 2012). The results I obtained during my thesis add information on the contribution of proliferation and neurogenesis to the process of eversion of the zebrafish pallium and complete this model of eversion.

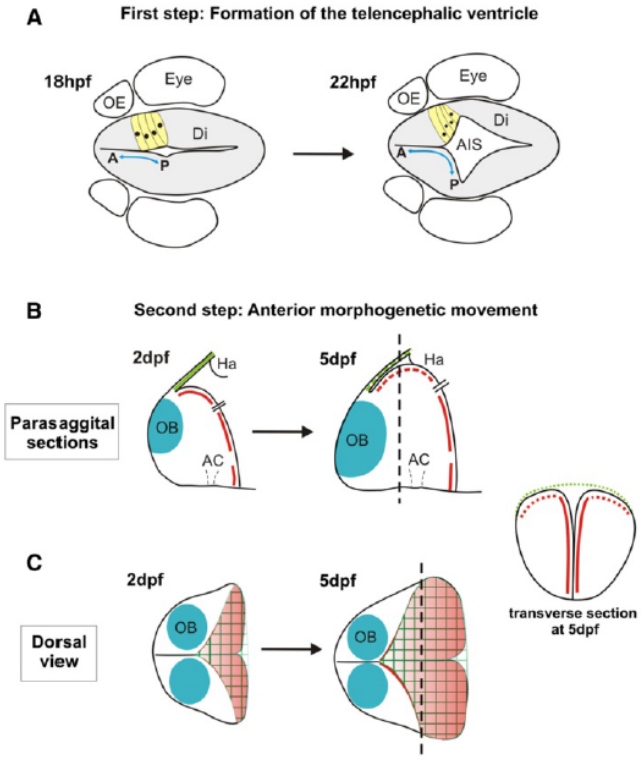


Figure 13: Two steps in zebrafish telencephalic morphogenesis.

Summary diagrams illustrate the major morphogenetic movements leading to the everted telencephalon in the zebrafish brain. A. First, at around 18 to 22 hpf, an out-pocketing of the ventricular surface forms the anterior intraencephalic sulcus (AIS) with its diamond-shaped roof. This fold forms the posterior wall of the telencephalic lobes and relocates the most posterior telencephalic territory to a more lateral position. B and C. Next, between 2 dpf and 5 dpf, the pallial domain expands along the AP axis, and the posterior wall of the telencephalon bulges into the ventricular space of the AIS. During this phase, the roof of the AIS (green) also expands along the AP axis to form the tela choroidea. The ventricular surface (red) of the dorsal AIS also bulges into the ventricular space and expands forwards over the upper surface of the telencephalon in close apposition to the tela. These rearrangements of the OB, tela choroidea, and posterior wall of the telencephalon between 2 dpf and 5 dpf are illustrated in diagrammatic parasagittal sections in B and dorsal view in C. The transverse section shows telencephalon has acquired its typical everted morphology at 5dpf. A; anterior; AC: anterior commissure; AIS: anterior intraencephalic sulcus; Di: diencephalon; Ha: habenula; OB: olfactory bulb; OE: olfactory epithelium; P: posterior.(Folgueira et al., 2012)

2 The telencephalon, a territory of continuous neurogenesis

The main targets of the patterning signals mentioned above are the “neural progenitors”. The latter correspond to the cells involved in the generation of differentiated cell types such as the neurons and glial cells. In theory, these likely regroup different populations of cells. First, the neural stem cells (NSCs) correspond to a particular population of cells that display self-renewing potential and multipotentiality. Second, proliferating progenitors, possibly generated by the NSCs, do not display any long-term self renewing capacity and are often dedicated to the production of one particular cell type.

In the vertebrate embryonic brain, actively dividing and neurogenic embryonic neural progenitors are involved in neuronal and glial generation during early brain construction. The long-term capacities of these progenitors have generally not been tested. In the adult brain, aNSCs, mainly found quiescent, are present in some areas such as in the mammalian hippocampus or the entire ventricular domain of the adult zebrafish telencephalon, and participate in the generation of adult-born neurons involved in physiological and behavioral modulations. Both embryonic neural progenitors and aNSCs can undergo different types of cell divisions, symmetric or asymmetric, depending on whether distinct cell types (ie. differentiated cells and/or progenitors) arise or not from the division. Interestingly, embryonic progenitors are located in the entire ventricular zone of the presumptive pallium, and aNSCs are present in some adult pallial regions in the mouse and in the entire adult pallial ventricular zone in the zebrafish, both deriving from the embryonic pallial germinal zone; even though the relationship between the embryonic neural progenitors and aNSCs is still unclear, this suggests that continuous neurogenesis may occur at least in some regions of the telencephalon in both mammals and fish.

In this second main section, I will address the issue of the neural progenitor, dealing first with embryonic neurogenesis in mammals and zebrafish, and then addressing the concept of aNSCs and adult neurogenesis. I will largely focus on the telencephalon, but use examples elsewhere when necessary. I will also introduce what is known of the different steps that enable the emergence of aNSCs through development.

2.1 Identity, maturation and fate of embryonic neural progenitors

During vertebrates CNS development, uncommitted progenitors are initially present in the neuroepithelium and undergo successive steps of maturation that progressively restrict their fate. Patterning signals, ie. positional values, impact both the degree and the timing of neural

progenitors amplification and contribution to neuron and glia generation by triggering changes into cell division modes and parameters, as well as the identity of their generated differentiated progeny by inducing different neurogenic programs.

At the population level, the progenitors acquire on the one hand a “neurogenic competence” that endows them with the capability to produce neurons, and on the other hand generate various differentiated glial or non-glial cell types such as astrocytes, oligodendrocytes, and ependymal cells in mammals. In addition, they give rise to a small population of self-renewing cells (NSCs) that will participate in the establishement of aNSCs pools. aNSCs are mainly composed of glial cells, often with radial glial-like features. In this section we will summarize what is known of the different processes that trigger the maturation of embryonic neural progenitors during development to achieve a proper brain construction. We will mainly focus on the telencephalon but information related to other systems will be added when required.

2.1.1 Cortical neurogenesis and progenitor maturation

At the onset of neural plate specification, epithelial ectodermal cells change and adopt a neural identity, thus becoming neuroepithelial cells (NE) (Götz and Huttner, 2005). They conserve epithelial features such as an apico-basal polarity, with the presence of tight and adherens junctions at the most apical end of their lateral plasma membrane (Götz and Huttner, 2005). After the formation of the neural tube, these cells are in contact with both the pial surface at their basal pole and the ventricle apically.

The neuroepithelium looks layered (or “pseudo-stratified”) due to interkinetic nuclear migration (Figure 14A). This process is linked with cell cycle progression (Götz and Huttner, 2005). Indeed, during interkinetic nuclear migration, in which both microtubules and actin filaments are involved, nuclei in S phase are located at the basal side of NE cells, while cells undergoing mitosis divide at the apical pole, close to the ventricle. Nuclei of cells in G1 or G2 are positioned at mid-region (Kriegstein and Alvarez-Buylla, 2009). Based on studies in the zebrafish retina, the interkinetic nuclear migration has been shown to be involved in regulating neurogenesis as an apico-basal gradient of Notch signaling, the main signalling pathway involved in regulating neurogenesis and embryonic neural progenitor maintenance (see section 3.1.2), is present in NE cells (Del Bene et al., 2008); as nuclei move within zebrafish retinal progenitors, they are thus differentially exposed to Notch signalling, influencing their fate decisions (Del Bene et al., 2008).

As embryonic neurogenesis progresses, the thickness of the epithelium increases and new progenitors appear, the radial glial cells (RGCs). They derive from NE cells and acquire a radial morphology, with their cell bodies located along the VZ and the appearance of an

elongated pial-directed radial process (Figure 14B and Figure 16) (Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). In addition to Nestin expression already present in the NE cells, RGCs express glial markers such as *GLAST*, *BLBP*, *GFAP* and *vimentin*, and make specific contact with the developing cerebral vasculature (Misson et al., 1991; Takahashi et al., 1990). The RGCs keep several features of NE cells such as the adherens junctions, the interkinetic nuclear migration and the apico-basal polarity (Figure 14B) (Kriegstein and Alvarez-Buylla, 2009), but contain glycogen granules, a feature of astrocytes. In the mammalian cortex, lineage experiments using *BLBP:Cre* mice indicate the transition between NE and RGCs starts approximately between E10.5 and E12.5 and is progressive up to E16.5 (Anthony et al., 2004). Embryonic RGCs are the progenitors of a large number of neurons in the entire brain, highlighting that RGCs are the main source of neuronal production during development (Figure 16) (Anthony et al., 2004).

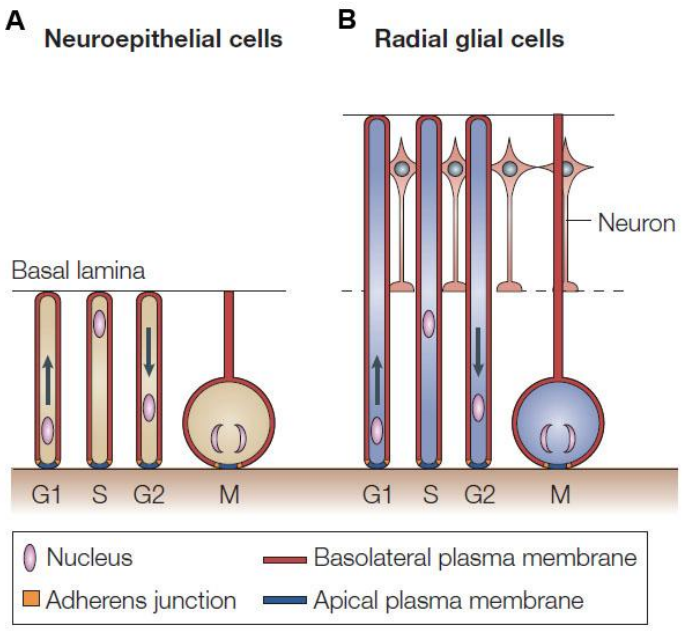


Figure 14 : Polarized features and interkinetic nuclear migration (INM) of neuroepithelial cells and radial glial cells in mammals

A: In Neuroepithelial (NE) cells, an apico-basal polarity is present with adherent junctions at the apical pole and a specialized apical membrane domain (blue). Interkinetic nuclear migration spans the entire apical–basal axis of the cell, with the nucleus migrating to the basal side during G1 phase, being at the basal side during S phase, migrating back to the apical side during G2 phase, and mitosis occuring at the apical surface. B: Radial glial cells (RGCs) have an apico-basal polarity as well with adherent junctions, and the basally directed interkinetic nuclear migration does not extend all the way to the basal side (that is, through the neuronal layer to their pial end-feet), but is confined to the portion of the cell between the apical surface and the basal boundary of the ventricular zone or the subventricular zone (not shown).
(Götz and Huttner, 2005)

Even though the vast majority of embryonic neurons are produced by the RGCs, the NE cells correspond to the first neural progenitors involved in neurogenesis. Indeed, they express progenitor markers such as *Nestin* and generate the first neurons of the central nervous system (Götz and Huttner, 2005). During the first phase of CNS development, NE cells mainly perform symmetric divisions. First, amplifying symmetric divisions occur, which participate in the growth of the neural tube. Then, neurogenesis starts in the neurepithelium due to the induction of proneural genes involved in the induction of the neurogenic program (see section 3.1.2).

The transition between NE and RGCs is also correlated with a transition from symmetric to asymmetric divisions with RGCs mainly producing one neuron and one RGC by a self-renewing division. It has been shown that *Sox1-3* are expressed in NE cells (Bylund et al., 2003) and *in vitro* experiments performed in ES cells indicate that it prevents NE cells from becoming RGCs (Suter et al., 2009). On the contrary, *Pax6* expression is restricted to the RGCs and forced expression of *Pax6* in NE cells triggers their transition toward RGC progenitors. *Pax6* mutant mice display a reduction of the RGCs neurogenic potential without affecting neurogenesis in NE cells, thus indicating that *Pax6* is involved in promoting the emergence of RGC progenitors and asymmetric cell divisions in this population (Heins et al., 2002; Suter et al., 2009). In addition, *Sox1* and *Pax6* seem to repress each other as overexpression of one of them triggers a dowregulation of the other (Suter et al., 2009), but whether this effect is direct or indirect remains to be determined. Similarly, we have already introduced that Pax6 and Emx2 are two factors involved in the patterning of the dorsal telencephalon that are able to repress each other, with Emx2 more specific of the caudo-medial pallium and Pax6 expressed in the rostro-lateral cells. *Emx2* expression promotes symmetric divisions: its overexpression in cortical progenitors leads to an increase in symmetric divisions and *Emx2* mutant mice analysed at E14 display a severe reduction of symmetric divisions in the cortex (Heins et al., 2001).

To conlude, NE cells are the first neural progenitors of the CNS, producing neurons following symmetric divisions; a switch between NE and RGCs occurs progressively during cortical development due to changes in genetic programs, and is linked with changes in cell division mode, with symmetric neurogenic or amplifiying divisions in NE cells, and asymmetric self-renewing divisions in RGCs.

As corticogenesis progresses, the SVZ emerges with IPCs and RGCs guide all the ventricular and subventricular produced neurons to generate the proper cortical layers. It has been shown that IPCs express specific transcription factors that will be found afterwards in cortical cell layers, such as Cux1 and Cux2 (Nieto et al., 2004; Zimmer et al., 2004). More recently, *Cux2* has been found in a salt and pepper expression pattern in the RGCs (Franco

et al., 2012), suggesting that the ventricular progenitor population could be heterogenous regarding its contribution to the different neuronal cortical layers.

2.1.2 Mitotic spindle orientation and asymmetric division

Asymmetric cell division can result from two different processes: either the asymmetric cell fate is determined before the cell divides, thus implying that some cell fate determinants were asymmetrically partitioned within the dividing cell, or it can arise from a post-division decision such as a differential Notch activation due to a “lateral inhibition-like process” occurring between the two daughter cells and generating activation of different genetic programs in the sister cells (see section 3.1.2.4). In the first situation, regulation of the mitotic spindle orientation influences the emergence of asymmetry in cell division. This asymmetry is formed during prophase when the centrosomes nucleate spindle microtubules to position the chromosomes and the spindle relative the cell cortex (Glotzer, 2009). The astral microtubules anchored to the cell cortex via their interaction with the sub-cortical F-actin network through the dynein-dynactin proteins can position the entire spindle in the cell relative to polarity cues.

A lot of the known factors involved in orienting the spindle relative to apico-basal polarity in vertebrates have been discovered in *Drosophila,* such as the LGN/NuMA/Gαi complex (in Drosophila: the Pins/Mud/Gαi) associated with dynein-dynactin at the spindle poles (Lancaster and Knoblich, 2012). In the case of a planar division, the complex is excluded from the apical side via its phosphorylation by aPKC. On the contrary, the presence of Insc allows the interaction of LGN with the apically localized Par3, thus triggering a vertical orientation of the mitotic spindle (Lancaster and Knoblich, 2012). Orientation of the mitotic spindle is thus important in Drosophila neuroblasts as it asymmetricaly segregates different factors, such as the Notch inhibitor Numb, that triggers the differenciation of the daughter cell into neuron (Kang and Reichert, 2014).

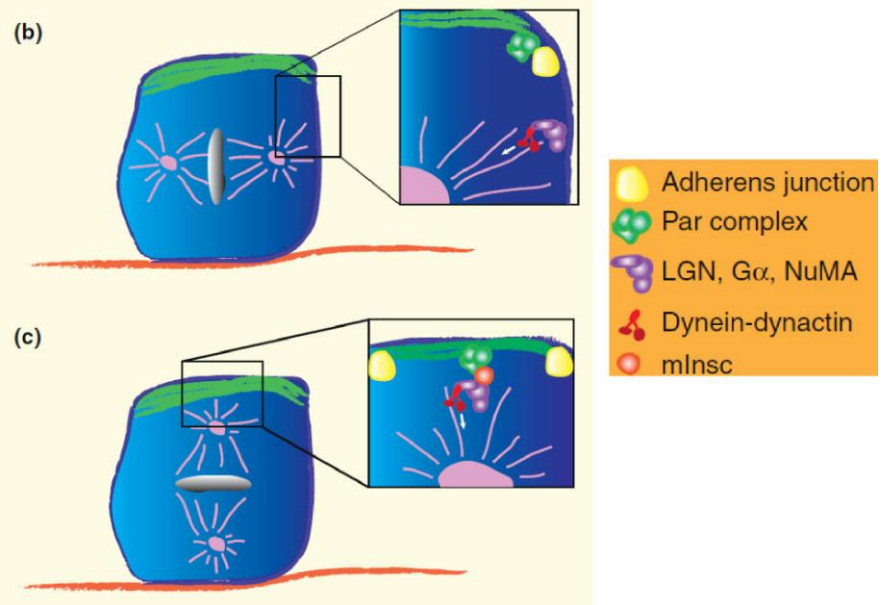


Figure 15: Spindle orientation during cell division in the mammalian epithelium

Planar division occurs when the spindle is positioned perpendicularly to the apicobasal axis (defined by the apical domain, green). This occurs through segregation of the LGN complex (purple) from the apical domain by Par complex proteins (green) and adherens junctions (yellow). Aster microtubules are then positioned through dynein–dynactin (red) association with the LGN complex. (c) Vertical orientation (along the apicobasal axis) occurs in the presence of mInsc, which allows association of the LGN complex with the Par complex. This connection pulls the spindle pole toward the apical domain, thereby orienting the spindle vertically. In all panels, the orange line marks the basal surface. Adapted from (Lancaster and Knoblich, 2012).

During mammalian corticogenesis, NE cells first divide symmetrically with a planar orientation of the mitotic spindle and this orientation is crucial to maintain the early progenitor population (Yingling et al., 2008). LGN is expressed in both NE and RGCs and has been shown to promote planar orientation of the mitotic spindle of the embryonic progenitors in the neocortex. Its loss leads to a randomization of the spindle orientation and an increase in IPC production at the expense of apical progenitors; but interestingly, it does not affect neurogenesis indicating that most spindle orientations lead to the generation of neurons and that only few configurations of the spindle orientation allow self-renewing divisions (Konno et al., 2008).

Interestingly, Inscuteable factor *(mInsc)* is expressed in the developing cortex at the emergence of RGCs and asymmetric divisions. Neither its dowregulation nor its overexpression affect early neurogenesis (Postiglione et al., 2011; Zigman et al., 2005), but, later, its overexpression leads to an increase in oblique and vertical spindle orientations whereas its deletion results in decreased oblique divisions and neurogenesis without affecting the number of RGCs. (Postiglione et al., 2011). As oblique divisions contribute to

neurogenesis via generating intermediate progenitors (IPCs), these results highlight that mInsc promotes IPCs production and thus amplify neurogenesis. During corticogenesis, the non-planar orientation of the mitotic spindle is thus important for the amplification of neuronal production, via the generation of IPCs.

The question of cell fate decisions is prominent in the case of asymmetric cell divisions. We already introduced that the role of Insc is to oriente the spindle by interaction with LGN and the apically located Par3 protein. Interestingly, like Drosophila neuroblasts, NE and RGCs are highly polarized cells and express *Par3* at their apical side in both mouse and zebrafish (Alexandre et al., 2010; Bultje et al., 2009; Manabe et al., 2002; Wei et al., 2004). Experiments performed in the zebrafish embryo by Alexandre and colleagues reveal unexpected results concerning the inheritance of the apical domain and cell fate. Using live imaging, these authors showed that during an asymmetric division in the caudal hindbrain/anterior spinal cord between 20 and 30hpf, the daughter cell inheriting the Par3-positive apical domain will become a neuron while the basal progenitor inheriting the basal process and only part of the junctional domain stay as a progenitor (Alexandre et al., 2010). Moreover, *par3* morpholino injections increase the proportion of symmetric self-renewing RGC-generating divisions, indicating that Par3 inheritance is necessary for asymmetric neurogenic divisions in the early zebrafish neural tube and is involved in determining the neuronal fate (Alexandre et al., 2010). These results were confirmed in the embryonic chick spinal cord (at HH10-12) in which the apical-inheriting daughter cell becomes a neuron whereas the basal daughter cell rapidly reestablishes an apico-basal polarity (Das and Storey, 2012). However, in mammals, inhibition of *Par3* in cortical progenitors at E14 via shRNA electroporation leads to an increase in symmetric neurogenic divisions. This leads to a progressive progenitor depletion (Bultje et al., 2009), indicating that, in the mammalian cortex, Par3 is necessary for asymetrical self renewing divisions. Moreover, it regulates the asymmetrical inheritance of Numb that inhibits Notch signalling in the inherited-basal daughter cell, thus triggering its commitment (Bultje et al., 2009). The results obtained in the zebrafish/chick and mammals seem to be contradictory; however, this could be due to temporal or regional differences, or to species-specific features. Moreover, in the mouse, these analyses were performed at the population level and more precise studies would be needed to determine whether such a role of Par3 is present in the cortex as well. This highlights that components differentially localized between apical and basal sides of the progenitor influence cell fate during asymmetric division.

Finally, it is interesting to note that centrosome inheritance is also important for the maintenance of the radial glial progenitors during corticogenesis. Indeed, Wang and colleagues have shown that, during the peak of neurogenesis in the cortex, there is an asymmetric inheritance of the centrosome with RGCs always keeping the old centrosome

and the neuronal progeny taking the duplicated version. Disrupting this process leads to a depletion of the progenitor population and they showed that some protein in the pericentrolar material segregate differentially between the initial centrosome and the duplicated one, indicating the potential role of some pericentriolar factors in progenitor maintenance (Wang et al., 2009). Moreover, it has been shown that the more mature centriole usually grows a primary cilium first (Anderson and Stearns, 2009), allowing for example quick Shh signal in the cell inheriting the mature centriole. This asymmetric centrosome inheritance would thus confer different sensitivities of the two daughter cells to external cues.

2.1.3 Radial glial lineage heterogeneity

At the end of cortical development, most cortical RGCs lose their ventricular attachment and migrate toward the cortical plate by a process of somal translocation. Their morphology changes from bipolar to unipolar that no longer contacts the ventricle, to multipolar with a regression of the radial process, and then they become an astrocyte (Kriegstein and Alvarez-Buylla, 2009). Indeed, Noctor and colleagues using retroviral labeling and time lapse imaging, have demonstrated that RGCs that had produced neurons can transform into astrocytes (Noctor et al., 2008). Evidences for the implication of epigenetic regulation in this process have been obtained. Indeed, at early stages, the promoters of the *GFAP* and *S100β* genes are methylated; later, RGCs only become competent to respond to differentiation factors via demethylation of the *GFAP* and *S100β* promoters. Signaling factors, such as Fgf2 acting through the nuclear co-repressor NCOR are involved in keeping gliogenesis inhibited in early progenitors (Hermanson et al., 2002; Molné et al., 2000; Takizawa et al., 2001).

Some astrocytes divide locally before terminal differentiation and represent a population of astrocytic intermediate progenitors (aIPCs). This allows the amplification of the population of astrocytes present in the cortex (Kriegstein and Alvarez-Buylla, 2009). Interestingly, astrocytes take a cortical position that mirrors the inside-out laminar birthdate of the neurons with the later-born astrocytes taking up superficial cortical positions (Ichikawa et al., 1983).

In addition to astrocytes, embryonic progenitors produce oligodendrocytes from different locations in the telencephalon that emerge during three different formation waves (Figure 16). A first wave of oligodendrocyte precursors is produced at a very early stage of development (E9/10) (Delaunay et al., 2008; Spassky et al., 1998). Later, a second wave of OPC production occurs in the ventral telencephalon from the MGE (*Nkx2.1*-positive precursors) and the LGE (*Gsh2*-positive precursors) domains at E14. The third wave arises at E18 from the dorsal cortex (*Emx1*-positive precursors) (Dimou and Götz, 2014). The first populations of OPCs disappear at birth and the late dorsal-originating oligodendrocytes will

compose the majority of the adult cortical oligodendrocytes (Kessaris et al., 2006). Oligodendrocyte precursors expressing *NG2* (OPCs or oIPCs) persist in the brain and can be reactivated upon local signals. These OPCs are likely derived from both RGCs and the adult SVZ (Gonzalez-Perez and Alvarez-Buylla, 2011; Kriegstein and Alvarez-Buylla, 2009).

Experiments based on retroviral labelling at mid-corticogenesis have also shown that cortical progenitors can generate clones with either astrocytes only, oligodendrocytes only, or both suggesting the existence of progenitors restricted to one glial cell type production and a few with bi-potentialities during development (Levison and Goldman, 1993; Luskin, 1994; Luskin et al., 1993). These observations highlight the heterogeneity of the ventricular progenitors that seems to be similar in terms of architecture and morphology but can be specialized in producing a restricted number of differentiated and functional cells.

Finally, RGCs generate the ependymal cells that line the ventricle (Figure 16). These cells are multi-ciliated and involved in driving the cerebro-spinal fluid (CSF) flow. They are post-mitotic and are generated from late RGCs that undergo their terminal division between E14 and E18 (Guérout et al., 2014). They acquire their mature features only during the first postnatal week. Different subtypes of ependymal cells (tanycytes, cuboïdal, radial ependymal cells) are reported depending on molecular markers, morphology and location but their origin and functionnal differences are still largely unknown (Guérout et al., 2014). Recently, *FoxJ1* and *Six3* have been shown to play a role in their specification and maturation (Jacquet et al., 2009; Lavado and Oliver, 2011).

In the zebrafish, neither astrocyte nor ependymal cell have been reported in the telencephalon, thus the transition from RGCs to astrocytes or from RGCs to ependymal cells never occurs. RGCs persist into adult life at the ventricular zone in the telencephalon (see section 2.2.3). However, telencephalic oligodendrocytes are produced during development and are present together with OPCs in the adult parenchyma of both pallium and subpallium and at the subpallial VZ (März et al., 2010a).

Interestingly, this progenitors sequence, based on the lineage tracing of radial glia and intermediate progenitors and BrdU labelling, consists in a linear view of how progenitors change during development and implies that progenitors described in the embryo are submitted to a series of maturation steps until adult stage. Nevertheless, we cannot exclude that some discrete populations of embryonic progenitors escape these maturation events and are still present in the adult. Some of the results I obtained during my thesis highlight that some embryonic NE progenitors persist the adult zebrafish pallium.

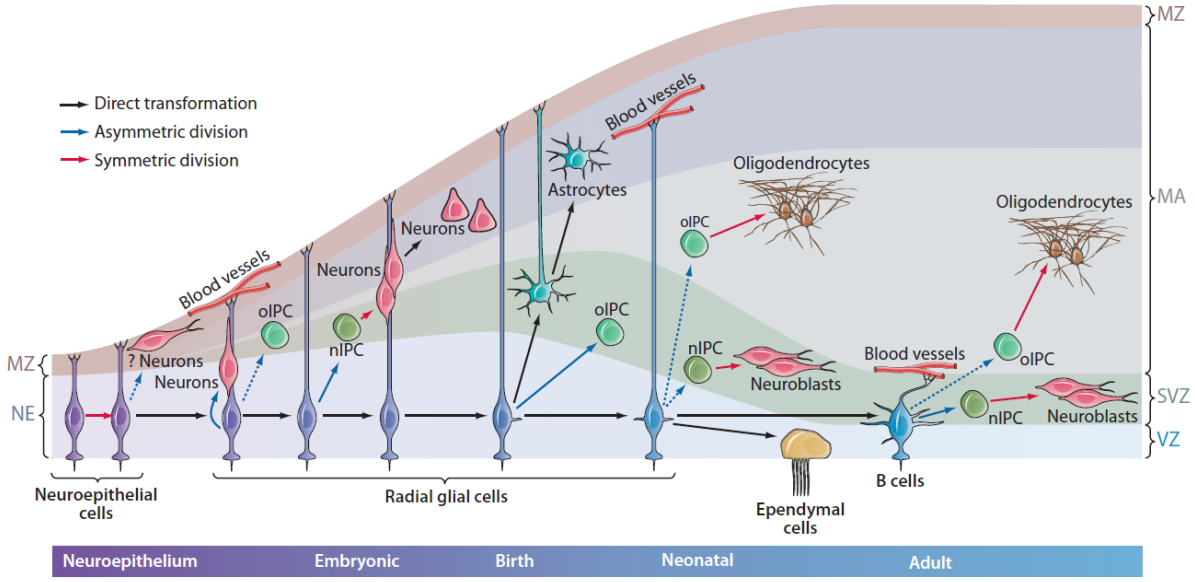


Figure 16: Neurogenesis and gliogenesis from the embryo to adult

Glial nature of neural stem cells (NSCs) in development and in the adult. This illustration depicts some of what is known for the developing and adult rodent brain. Timing and number of divisions likely vary from one species to another, but the general principles of NSC identity and lineages are likely to be preserved. Solid arrows are supported by experimental evidence; dashedarrows are hypothetical. Colors depict symmetric, asymmetric, or direct transformation. IPC, intermediate progenitor cell; MA, mantle; MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic progenitor cell; oIPC, oligodendrocytic progenitor cell; RG, radial glia; SVZ, subventricular zone; VZ, ventricular zone. From Kriegstein and Alvarez-Buylla, 2009

In the frame of this work, the notion of maturation of the embryonic neural progenitor is fundamental as lineage tracing experiments allowed to appreciate the different states of a progenitor population along development. Some of the results I obtained during my thesis give new information on progenitor maturation with a much less linear view than it has been proposed so far.

2.2 Adult neural stem cells - neurogenesis

Stem cells are defined as a group of cells that, via undergoing symmetric and asymmetric divisions, are capable at a single cell level of a prolonged self-renewal and of differentiation into several specialized cell types, responsible for physiological functions. Under physiological conditions in systems with slow renewal (muscle, brain…), they are mainly found in cell cycle arrest, defined as a quiescent state, but can become activated and re-enter the cell cycle upon stimulation. During the last two decades, stem cells have been extensively studied in the vertebrate brain, such as in the mouse or zebrafish, and even in human, where the presence of adult neurogenesis has been recently clearly demonstrated (Ernst et al., 2014; Spalding et al., 2013). In the case of the central nervous system, it is interesting to note that aNSCs are unipotent according to the "stricto sensu" stem cell definition, since most of them only generate neurons, but can be considered mutipotent regarding the different subtypes of neurons they can produce. The other main feature of the stem cell definition is self-renewal; however, this capacity at the single cell level is difficult to appreciate and has only been tested so far using neurosphere assays, in which a putative neural stem cell is isolated and cultured, and where self-renewal is assessed by measuring the number of possible passages before the cell exhausts, and multipotency is assessed via a differentiation paradigm (Pastrana et al., 2011). However, this technic does not allow to access the properties of quiescent stem cells, nor to reliably appreciate cell properties under physiological conditions. Moreover, tissue homeostasis can involve a second aspect of self-renewal, which occurs at the population level with some progenitors compensating by symmetric division for the terminal differentiation of others (Simons and Clevers, 2011). Finally, all these aspects can change over time and are probably different in physiological or pathological conditions -after brain lesions for example- (Dimou and Götz, 2014), highlighting that the stem cells adapt also to their environnement. Thus, the analysis of adult stem cells should now integrate the state of the environnement on the one hand and the population behavior on the other hand. In the following section, we will briefly introduce how adult neurogenesis was discovered, then the general features of adult neurogenesis in mammals, and finally, we will address the state of art about adult neurogenesis and aNSCs in the zebrafish.

2.2.1 Discovery of adult neurogenesis – cooperation of birds and rodents

Even though stem cells are found almost everywhere in the body, it was firmly believed until less than two decades that the adult brain was too complex to incorporate adult-born neurons. The neurobiologists Joseph Altman and Gopal D.Das in 1960s had observed a neurogenic activity in the post-natal hippocampus and olfactory bulbs of rats and guinea-pigs via tritiated thymidine injections (Altman, 1969; Altman and Das, 1965, 1967), but their work

was largely ignored. Ten years later, Kaplan and Hinds, by combining electron microscopy and tritiated thymidine staining, showed adult-born neurons in the dentate gyrus and the olfactory bulb of rats (Kaplan and Hinds, 1977). Then, adult neurogenesis was observed in the avian brain (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984). At the end of the 1980s already, *in vitro* experiments and clonal analyses via virus infections introduced the concept of "neural stem cell-like" cells that can produce both neurons and glial cells in the embryonic rodent brain (reviewed in Gage and Temple, 2013). Then, following observation of cell cultures collected from adult brain structures, the notion of aNSCs emerged (Gage et al., 1995; Lois and Alvarez-Buylla, 1993; Reynolds and Weiss, 1992). Together, these experiments led to the current model where adult neurogenesis in rodents constitutively occurs into two telencephalic regions: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, and the subependymal zone (SEZ) of the lateral ventricle. In addition to the telencephalon, adult neurogenesis has been also reported in the hypothalamus (Migaud et al., 2010), the striatum (Luzzati et al., 2007) and the rabbit cerebellum (Ponti et al., 2010).

Eriksson and colleagues, via Bromodeoxyuridine (BrdU) incorporation in a group of cancer patients, have been the first to find that adult neurogenesis occurs also in the human hippocampus (Eriksson et al., 1998). More recently, clever experiments based on the post-mortem analysis of brains of people who had eaten plants (or animals fed from these plants) exposed to an environnemental elevation of ^{14}C due to the nuclear bombs tests during the cold war (1955-1963), has confirmed that adult neurogenesis occurs in the SGZ. Concerning SEZ neurogenesis, compared to the situation in rodents, it generates neurons in the striatum and does not seem to contribute to the olfactory bulbs in the human brain (Ernst et al., 2014; Spalding et al., 2013).

2.2.2 Mouse adult neural stem cells and neurogenesis

In the mammalian brain, neurogenesis occurs in the SGZ of the hippocampus, in which new-born neurons integrate into the granule cell layer, and the SEZ of the telencephalic lateral wall, from which neuroblasts migrate along the rostral migratory stream and generate interneurons that integrate into the olfactory bulbs (Figure 17) (Zhao et al., 2008). In these two neurogenic zones, and at the population level, aNSCs can self-renew and differentiate into several neural cells such as particular types of neurons, astrocytes, and oligodendocytes (Dimou and Götz, 2014).

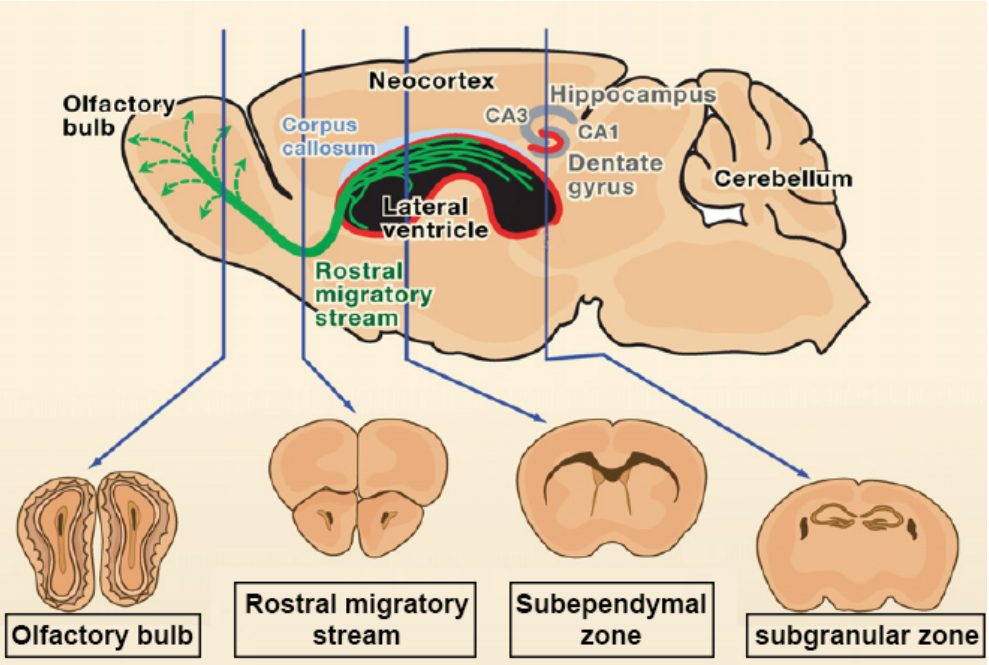


Figure 17: Adult neurogenenic zones in rodents

Schemes of sagittal and coronal views of the mouse adult brain in areas where neurogenesis occurs. Red areas indicate the germinal zones in the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subependymal zone (SEZ) of the lateral ventricles. Neurons generated from the SEZ migrate through the rostral migratory stream and are incorporated into the olfactory bulbs. Adapted from (Zhao et al., 2008)

2.2.2.1 The subgranular zone (SGZ) of the hippocampus

In the SGZ, several types of neural progenitors can be identified, according to morphologies and their expression of unique sets of molecular markers. The first class (Type 1 cells) of progenitors has a radial process spanning the entire granule cell layer and ramified in the inner molecular layer. Type 1 cells express stem cells markers such as the Sry-related HMG box transcription factor Sox2, and are rarely dividing cells (Suh et al., 2007). Despite their expression of the GFAP (glial fibrillary acidic protein) and Nestin glial markers, they are morphologically and functionally different from mature astrocytes and resemble more RGCs (hence called radial glia-like, RGLs). Interestingly, they are labelled by expression of the Notch target gene *Hes5* (Zhao et al., 2008). The second class corresponds to a non-radial population (Type 2 cells) and contains rapidly dividing progenitors expressing, like Type 1 progenitors, the markers *Sox2* and *Hes5* (Lugert et al., 2010; Suh et al., 2007). Lineage tracing experiments suggested that both Type 1 and Type 2 *Sox2*-positive progenitors display aNSCs properties (Suh et al., 2007). Nevertheless, the precise lineage relationship between these progenitors has not been clearly determined and several hypotheses concerning their hierarchy have been elaborated (Figure 18A) (Bonaguidi et al., 2012). One

possibility is that radial Type 1 cells generate the Type 2 cells (Bonaguidi et al., 2011; Dranovsky et al., 2011; Encinas et al., 2011). In this model, Type 1 cells are multipotent and can generate both glial and Type 2 cells. The latter are unipotent and generate neuroblasts that differenciate into neurons. There, two models exist: one considering that Type 1 cells can self-renew by asymmetric divisions (Figure 18A – left panel) (Dranovsky et al., 2011), and the other in which once Type 1 cells are engaged into neurogenesis, they terminate at some point their lineage and thus differentiate into astrocytes (Figure 18A- middle panel) (Encinas et al., 2011). The latter hypothesis suggests the existence of a "cell division clock" that progressively depletes the pool of progenitors as adult neurogenesis progresses.

Yet, another possibility is that the Type 2 cells are at the top of the hierarchy. In this case, they would generate on the one hand a reservoir of Type 1 NSCs that rarely divide, and on the other hand astrocytes and neurons without going through a radial glial state. Type 1 cells would thus generate only non-radial *Sox2*-positive cells that are able to produce both neurons and astrocytes (Figure 18A – right pannel) (Suh et al., 2007). Further studies are thus needed to address the mechanisms underlying progenitors hierarchy. In any case, all these models converge toward the production, from Type 2 cells, of actively dividing neuroblasts that are considered as secondary transit amplifying precursors (Figure 18B) (Mu and Gage, 2011). They first express *Tbr2*, and further *Doublecortin* (*DCX*) and *Prox1* that are considered as markers for immature neurons (Bonaguidi et al., 2012). These neuroblasts then differentiate into glutamatergic dentate granule cells that go through several steps of maturation during which their properties, such as their response to GABA, and morphologies, such as the density of mushroom spines, change. Their survival and integration in the SGZ are largely determined during a critical time window (1-3 weeks after birth), and this decision is influenced by the animal's experiences (Zhao et al., 2008). It is worth noting that oligodendrocytes do not seem to be generated under physiological conditions in the SGZ; however, it has been shown that SGZ aNSCs retain oligodendrocyte generation capacities. Upon for example overexpression of *Mash1* using retroviral infections in the SGZ, oligodendrocytic-cells are produced and incorporate durably in the dentate dyrus (Jessberger et al., 2008).

Astrocytes are also found in the niche and it has been suggested that they could play an important role in SGZ neurogenesis stimulation through Wnt signaling pathway (Lie et al., 2005; Song et al., 2002).

All the experiments based on mutant analyses or environmental changes lead to an increase in SGZ neurogenesis, via an action either at the proliferation level or at the level of the survival of the new-born neurons, and improve learning and memory capacities. Conversely, decreasing SGZ proliferation is correlated with an impairment in spatial memory and its consolidation or with defective learning (Zhao et al., 2008). In aged animals, which display a

decreased proliferation and neurogenesis correlated with an impairment of learning and memory, increasing SGZ neurogenesis either by voluntary activity (running) or by environmental enrichment rescues these hippocampal capacities (Zhao et al., 2008). Interestingly, recent work revealed that adult neurogenesis modulates forgetting. Indeed, Akers KG and colleagues, using both adult and infant mice, showed that increasing neurogenesis via running, genetic techniques or pharmacological treatments promotes forgetting (Akers et al., 2014). Adult neurogenesis in the SGZ in thus really involved in modulating adult hippocampal functions.

Finally, in addition to a physiological role of neurogenesis, it is emerging that aNSCs play also non-neurogenic functions. Indeed, in the SGZ, apoptotic newborn cells are cleared out through phagocytosis by microglia, cells responsible for active immune defense in the brain, and adult progenitors are able to modulate microglial activity via VEGF secretion (Mosher et al., 2012; Sierra et al., 2010). Moreover, neuroblasts produced in the SGZ regulate stress activity at both endocrine and behavioral levels by buffering stress response through the regulation of the hypothalamic-pituitary-adrenal axis (Snyder et al., 2011).

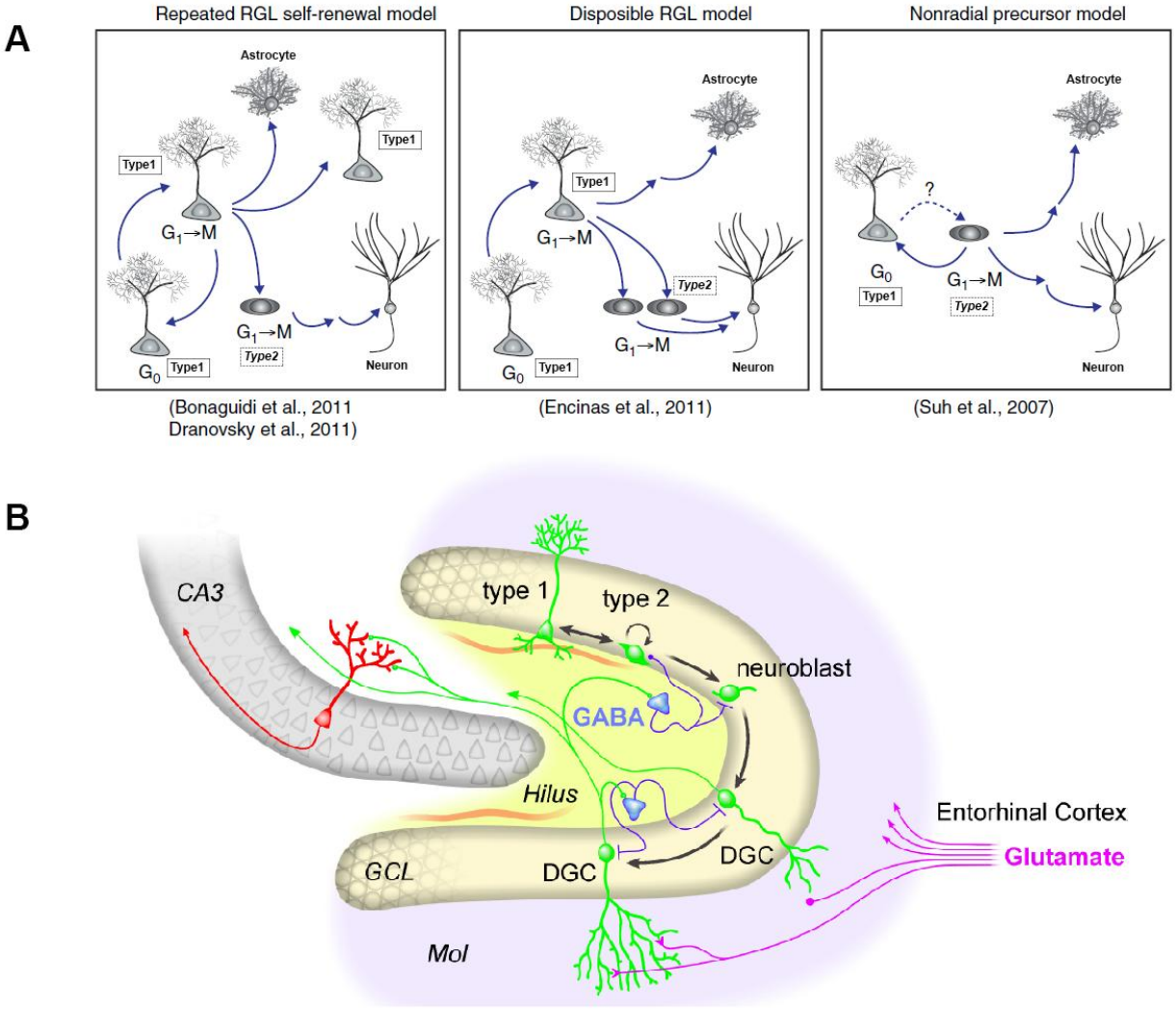


Figure 18: Adult neurogenesis in the SGZ of the mouse hippocampus

A: Three proposed NSC models. In the 'Repeated RGL self-renewal' model, RGLs can cycle between quiescent and mitotic states. Once activated, RGLs can divide symmetrically to generate additional RGLs, or asymmetrically to produce neuronal and astroglial lineages. In the 'Disposable RGL' model, once activated, RGLs repeatedly divide to generate only the neuronal lineage without returning to quiescence and then terminally differentiate intoastrocytes. In the 'Nonradial precursor' model, proliferative cells lacking a radial process generate neurons, astrocytes, and even RGLs. Arrows indicate direct cell generation. Dotted arrows represent unknown choices. Double arrows represent multistep cell generation. Adapted from (Bonaguidi et al., 2012)

B: The quiescent type 1 NSCs coexist with actively proliferating non-radial type 2 NSCs and they generate both astrocytes and neuroblasts. Neuroblasts migrate into the granule cell layer (GCL) and differentiate into dentate granule cells (DGCs). Newborn DGCs gradually develop and acquire progressively their mature properties. They elaborate dendritic trees in the molecular layer (Mol) to receive inputs from the Entorhinal cortex and project to CA3 pyramidal neurons (red) as well as interneurons located in the hilus (blue) (Mu and Gage, 2011)

2.2.2.2 The subependymal zone (SEZ)

The SEZ is located just underneath ependymal cells, a thin layer of multiciliated cells that lines the two lateral ventricles of the telencephalon and that contributes to the flow of the cerebro-spinal fluid (Figure 19) (Ihrie and Alvarez-Buylla, 2011). Ependymal cells are quiescent and whether they display stem cells properties is still unclear, as some ependymal cells reactivation and depletion have been observed after stroke (Carlén et al., 2009) but they do not seem to participate in adult neurogenesis under physiological conditions (Consiglio et al., 2004).

As for the SGZ, different types of progenitors constitute the NSCs niche in the SEZ. First, the **slowly proliferating progenitors, also named Type B** cells which can be divided into two categories depending on their location in the niche and their morphology: the Type B1, closely associated with ependymal cells and directly contacting the ventricle via an apical process containing a non-motile primary cilium, and the Type B2 cells, located close to the striatal parenchyma (Figure 19) (Ihrie and Alvarez-Buylla, 2011). Type B1 cells are connected both with other type B1 cells and ependymal cells on their apical part, and extend their basal process from the SEZ to reach the basement membrane surrounding blood vessels. They display a radial morphology and are glial cells as they express glial markers such as the *glutamate transporter* (*GLAST*), the *brain-lipid-binding protein* (*BLBP*), *GFAP*, *Nestin* and *Vimentin*. Prominin is used as a marker for stem cells and is expressed in both Type B1 and ependymal cells (Ihrie and Alvarez-Buylla, 2011). Type B1 cells are considered as the NSCs of the SEZ as, upon division, they produce the second type of progenitors in the SEZ, the **Type C cells, also referred to as the transit amplifying precursors (TAPs or IPCs**) (Figure 19) (Kriegstein and Alvarez-Buylla, 2009). These are located close to the Type B1 cells and express specifically *Mash1* and *Dlx2* (Ihrie and Alvarez-Buylla, 2011). The progeny of all these cells are **Type A cells, or neuroblasts**, which migrate toward the olfactory bulbs (Figure 19) (Lois and Alvarez-Buylla, 1994). They express, as the type C cells, *Dlx2* but also express specifically the immature neuronal marker *Doublecortin* (*D*cx) and the *Polysialyted Neural Cell Adhesion Molecule (PSA-NCAM)*, involved in the migration process (Chazal et al., 2000; Ihrie and Alvarez-Buylla, 2011). Several other molecules, such as Fascin, a factor involved in axonal growth, have been shown to be involved in the regulation of this migration process (Sonego et al., 2013).

It is now emerging that some of the different markers mentioned above would much more characterize a “state” of cell rather than a “type” of cell, and they are actually often not restricted only to one cell type. This highlights the concept of continuum between all these different categories of cells. For instance, contrary to *GFAP* expressed exclusively in type B cells, *GLAST* is also expressed in a small subset of type C cells (Pastrana et al., 2009). The same holds for *Mash1*, which is expressed, in addition to type C cells, in small number of

type B cells, possibly highlighting the Type B cells engaged into the soon generation of Type C cells (Kim et al., 2011).

Once the neuroblasts are produced, they migrate via the rostral migratory stream to arrive into the olfactory bulbs (Figure 19). It has been proposed that astrocytes and vasculature play an important role in this process via building a scaffold for fascilitating neuroblasts migration (Alvarez-Buylla and Garcia-Verdugo, 2002; Whitman et al., 2009). Almost one half of the adult-born neurons originating from the SEZ will survive and their elimination occurs, as for the SGZ, during a critical period (between 2-4 weeks after birth) (Yamaguchi and Mori, 2005). Adult SEZ neurogenesis produces inhibitory interneurons that either use GABA and dopamine as neurotransmitters (periglomerular neurons), or only GABA (granule cells), the latter representing 90% of the adult-born neurons produced by the SEZ (Gheusi and Lledo, 2014).

In terms of gliogenesis, under physiological conditions, the production of oligodendrocytes is scarce in the SEZ with only a subpopulation of Type B and Type C cells that express the *Oligodendrocyte lineage transcription factor 2* gene (*Olig2*) (Menn et al., 2006). This population of oligodendrocytes precursors migrate into the copus callosum, the striatum and the fimbria fornix to differentiate into *NG2*-positive cells that continue to divide locally and mature into myelinating oligodendrocytes (Menn et al., 2006). The migration and differenciation of OPCs is regulated by *endothelin-1*, an astrocyte-derived signal (Gadea et al., 2009). However, whether *Olig2*-positive precursors and IPCs derive from the same Type B cells at the single cell level remains to be determined; clonal analyses would be necessary to address this question.

In terms of functional relevance, SEZ neurogenesis has been proposed to play a role in olfactory perception and memory via its role on neuron survival, olfactory discrimination or pattern recognition, and olfactory memory (Gheusi and Lledo, 2014).

As in the SGZ, the stem cells of the SEZ also perform non-neurogenic functions, for example via neuroblasts that exert a phagocytic activity in clearing apoptotic neural precursors using proteins of the engulfment pathway (Lu et al., 2011).

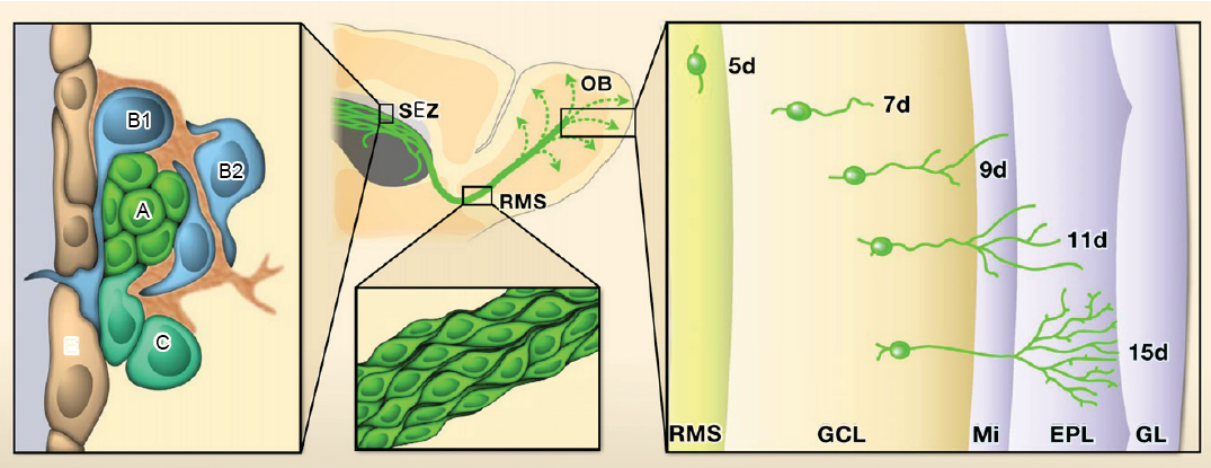


Figure 19: Adult neurogenesis from the SEZ to the olfactory bulb

Progenitor cells (A–C) in the subependymal zone (SEZ) lie adjacent to the ependymal cell (E) layer lining the lateral ventricles and interact with basal lamina extending from the local vasculature. Newborn neurons reach the olfactory bulb (OB) through chain migration and go through morphological and physiological development before integrating as granule neurons in the granule cell layer (GCL) and as periglomerular neurons (not shown) in the glomerular layer (GL). Abbreviations are as follows: Mi, mitral cell layer; EPL, external plexiform layer; RMS, rostral migratory stream.adapted from (Zhao et al., 2008)

2.2.2.3 The adult cerebral cortex

The adult cerebral cortex possesses parenchymal astrocytes; however, in a healthy brain, these do not divide. When isolated *in vitro*, they are not able to self-renew and to generate neurospheres, thus do not diplay stem cells properties (Buffo et al., 2008).

Another type of glial cells present in the parenchyma is the *NG2*-positive glia. Contrary to astrocytes, these cells proliferate slowly outside the stem cell niches in the adult brain. They generate oligodendrocytes and self-renew as they produce NG2-glia (Dimou and Götz, 2014). Even though their multipotency *in vivo* is controversial, studies *in vitro* have shown that when they are isolated from a postnatal brain hippocampus, they can give rise to mutipotent neurospheres with functional neurons, astrocytes and oligodendrocytes (Belachew et al., 2003).

Interestingly, after injury, these two types of progenitors react and change their behavior. *NG2* glial cells increase their proliferation right after the injury (Kang et al., 2010; Simon et al., 2011). Concerning the astrocytic reaction, also called astrogliosis, they become hypertrophic, and upregulate the glial markers GFAP and Vimentin, but also immature glial markers typical of RGCs such as Nestin and BLBP, and in some cases the NSCs marker Musashi (Robel et al., 2011). In severe trauma, they even start to proliferate and participate in the generation of the multiple astrocytes that are present at the site of lesion (Buffo et al., 2010; Sofroniew, 2009). Cultured *in vitro* after injury, they display stem cells features as they self-renew and generate neurospheres (Buffo et al., 2008; Lang et al., 2004). Genetic lineage

tracing experiments have shown that astrogliosis occurs in the mature parenchymal astrocytes (Buffo et al., 2008) and that one week after injury, half of the astrocytic pool has undergone cell division (Buffo et al., 2008; Simon et al., 2011).

It is worth noting that in injury conditions in the mammalian cerebral cortex, even though the lesionned region is not regenerated, neuroblasts are recruited at the injury site and can generate a few neurons such as glutamatergic projection neurons, that integrate into the neuronal network; however, these neuroblasts probably originate from the constitutive adult neurogenic regions (Robel et al., 2011).

2.2.3 Zebrafish neurogenesis and adult neural stem cells

Due to its evolutionary proximity with human, the rodent brain has been extensively studied in terms of adult neurogenesis, even though we start to discover that rodent and human adult neuronal production do not occur exactly in the same way (Ernst et al., 2014; Spalding et al., 2013). In the zebrafish, adult neurogenesis is prominent, and is more and more studied as we realize that it shares many common features with rodents.

2.2.3.1 Zebrafish adult neurogenesis

As mentioned previously, adult neurogenesis is not restricted to mammalian brains but is also present in birds, in which the process has been partially discovered (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984), and in reptiles, amphibians, and fish such as in the zebrafish (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006). In all these animals, adult neurogenesis is much more abundant than in mammals (Chapouton et al., 2007). It usually takes place in subdomains of ventricular zones of several brain areas and can serve in multiple adaptative functions (Chapouton et al., 2007).

In the zebrafish, using a combination of markers and BrdU incorporation experiments, 16 different loci of adult neurogenesis distributed throughout all brain subdivisions have been described, and especially in the telencephalon (Figure 20) (Adolf et al., 2006; Grandel et al., 2006; Pellegrini et al., 2007; Zupanc et al., 2005). In the latter, neurogenesis occurs at the level of the most superficial layer of cells lining the ventricle, all along both pallial and subpallial areas. Indeed, short lineage tracing of proliferating cells via Brdu pulse/chase experiments revealed that proliferating ventricular cells generate neurons that settle at just a short distance from the ventricular zone into the parenchyma (Adolf et al., 2006). This indicates that constitutive neurogenesis occurs in zebrafish in additional telencephalic regions compared to the rodent brain, such as the dorsal pallium (mainly Dd/Dc in fish), equivalent to the cortex in mammals where no constitutive neurogenesis has been reported, or in the ventral subpallium (Figure 20).

Moreover, adult-born neurons have been reported in the olfactory bulbs in fish as well (Adolf et al., 2006; Zupanc et al., 2005). These neurons show similarities with the ones produced by the mouse SEZ as they are GABAergic and dopaminergic (Adolf et al., 2006; Grandel et al., 2006). It has been proposed that these neurons derive from non-glial PSA-NCAM-positive proliferating progenitors located in the subpallium, the migration of which has been compared with the rostral migratory stream of mammals (Adolf et al., 2006; Grandel et al., 2006). A recent work showing, via live imaging, that subpallial *neurog1*-positive progenitors migrate and reach the olfactory bulbs along the vasculature also supports this model (Kishimoto et al., 2011). It is interesting to note that a similar RMS-like structure has been described in other vertebrates such as birds (Doetsch and Scharff, 2001) but does not seem to be present in humans (Ernst et al., 2014).

Finally, we discussed already that the field homologous to the hippocampus, the second neurogenic region in mouse, is considered to be located in the most lateral region of the zebrafish pallium (either Dl only, or Dl+Dp depending on the theory - see section 1.4). In this region as well, active proliferation and neurogenesis have been reported (Grandel et al., 2006; März et al., 2010b; Zupanc et al., 2005).

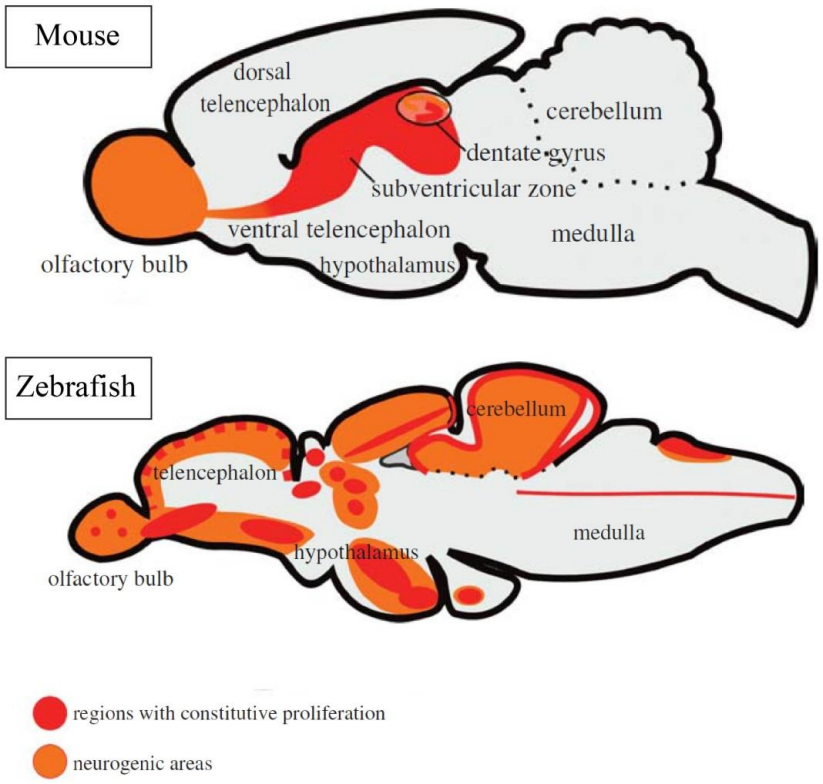


Figure 20: Parasagittal schematic overviews of the adult proliferation pattern and neurogenic regions in the brain of mammals and fish. From Kaslin et al., 2008

Compared to mammals, the zebrafish thus displays widespread neurogenesis that is involved in producing neurons all along life. What could be the role of maintaining such a number of neurogenic regions? The functional role of adult neurogenesis in zebrafish is still unknown; however, a few non-exclusive hypotheses could help us understand the potential of adult neurogenesis. First, the zebrafish grows continuously along life, and peripheral organs expand through the addition of new cells. Thus, new central neurons could be involved in reading these newly-formed peripheral organ regions. They could also be necessary for new behaviors. Second, at least in the lateral pallium, zebrafish adult neurogenesis could have a similar physiological role as in the mammalian hippocampus, with adult hippocampal neurogenesis involved in the spatial learning and memory (Zhao et al., 2008). Indeed, aging of the zebrafish leads to a decrease in proliferation and neurogenic activity (Edelmann et al., 2013) and an impairment of cognitive functions has been observed with aging (Yu et al., 2006b).

Interestingly, contrary to mammals, zebrafish display an extensive regenerative capacity (Gemberling et al., 2013), and aged zebrafish display a decreased regenerative capacity (Edelmann et al., 2013). This indicates that the maintenance of a large pool of aNSCs correlates with regenerative capacities.

2.2.3.2 Zebrafish adult neural progenitors

März et al performed a molecular description of the different progenitors located in the VZ "niche" (März et al., 2010b). Like for mouse progenitors, two main categories of progenitors are present along the adult telencephalic VZ: progenitors with a radial process and progenitors without a radial process. The first type of progenitors corresponds to Radial Glial Cells (RGCs) expressing GFAP, BLBP, Nestin, Sox2 and Glutamine synthase (GS) (Lindsey et al., 2012; März et al., 2010b). In the zebrafish telencephalic ventricular zone, RGCs are directly in contact with the ventricle; they express the ependymoglial marker S100β and are linked to each other via tight junctions (Figure 21)(Grupp et al., 2010; März et al., 2010b). It has been shown that these RGCs are ciliated cells that possess either a single cilium or multiple cilia depending on their location (Kishimoto et al., 2011; Lindsey et al., 2012). At least some of these ventricular RGCs self-renew and and are multipotent at a single cell level under physiological conditions (Rothenaigner et al., 2011). Finally, at a given time point, they can be subdivided into "quiescent" and dividing populations, the latter being defined by the expression of cell cycle markers such as PCNA or MCM5 (März et al., 2010b), which are present throughout most cell cycle phases (Barton and Levine, 2008). The "quiescent" RGCs, also called type I cells, represent 85% of the ventricular progenitors and proliferating RGCs at a given time point, while type II cells make only 8%. The current interpretation of this is that the vast majority of the RGCs population cycles very slowly or does not proliferate

(Figure 21) (Chapouton et al., 2010). BrdU pulse/chase experiments highlighted that, in the telencephalic ventricular zone, some RGCs that were proliferating two months before in an adult animal can re-enter the cell cycle and are thus long-lasting progenitors (Adolf et al., 2006). Whether, under physiological conditions, these cells constitute a subpopulation of RGCs with self-renewal capacity or whether all RGCs self-renew at some point in the adult telencephalic ventricular zone remains to be investigated. Interestingly, recent experiments have shown that most of the ventricular RGCs can become activated and engage into neurogenesis upon a treatment inhibiting the Notch signaling pathway, and can re-enter quiescence after treatment (Alunni et al., 2013; Chapouton et al., 2010), indicating that a large proportion of RGCs possess adult NSCs capacities.

The other progenitor subtype present in the zebrafish ventricular zone is the type III cell (Figure 21). This population expresses the proliferation marker PCNA, the neuroblast marker PSA-NCAM and the stem cells marker Sox2 (März et al., 2010b). These cells are thus considered as fast-proliferating neuroblasts in the zebrafish ventricular zone. They are mainly found in the RMS-like domain, as explained above, but they also represent 6% of the rest of the ventricular progenitors, indicating that they are also involved in pallial neurogenesis (Chapouton et al., 2010). As in the mouse system, it has been shown that some type III neuroblasts also express progenitor markers such as Nestin suggesting the presence of "transition state" progenitors generated from type II cells within this population (März et al., 2010b). Clonal lineage tracing experiments based on virus transduction targeting RGCs, together with experiments modulating the Notch pathway, have led to the current schematic view of neurogenesis in the zebrafish adult telencephalon: type I RGCs rarely enter the cell cycle, when they do so they become proliferating type II cells that divide and can either generate two RGCs (symmetric division – 86% of cases) and amplify the pool of NSCs, or one RGC and one type III/neuroblast (asymmetric division), that will further divide and differentiate into neurons (Figure 21) (Alunni et al., 2013; Chapouton et al., 2010; Rothenaigner et al., 2011). This situation strongly contrasts with the mouse in which the proportion is opposite with a majority of asymmetric divisions from aNSCs in both the SGZ and SEZ (Morshead et al., 1998; Suh et al., 2007). However, the rate of amplification at the neuroblasts level is still unclear but seems to be low in the zebrafish as clonal analyses always reveal a small number of neurons per clones (Rothenaigner et al., 2011). Genetic lineage tracing experiments designed to target specifically each of these different cell types would be necessary to really confirm the model.

Interestingly, in the zebrafish adult pallial germinal zone, like in the mouse, differences between RGCs have been observed in terms of the expression of glial markers, such as in the most lateral edge of the ventricular zone where some cells express BLBP but not S100β, but also morphologies with more or less ramifications of the radial process depending

on the spatial localization of the cell. This highlights a heterogeneity within the RGCs population that is not linked with the division state, as both quiescent and dividing cells where shown with these phenotypes (März et al., 2010b). Whether these differences reflect different degrees of maturation of the RGCs or other intrinsic features remains to be determined.

Overall, the different mammalian adult neurogenic niches are organized in different ways with specificities (see 2.2.2) whereas the telencephalic zebrafish germinal zone seems to be much more uniform. However, the results I obtained concerning the origin of the adult zebrafish germinal zone indicate that it is actually heterogenous with a mode of aNSCs formation that differs depending on the pallial regions.

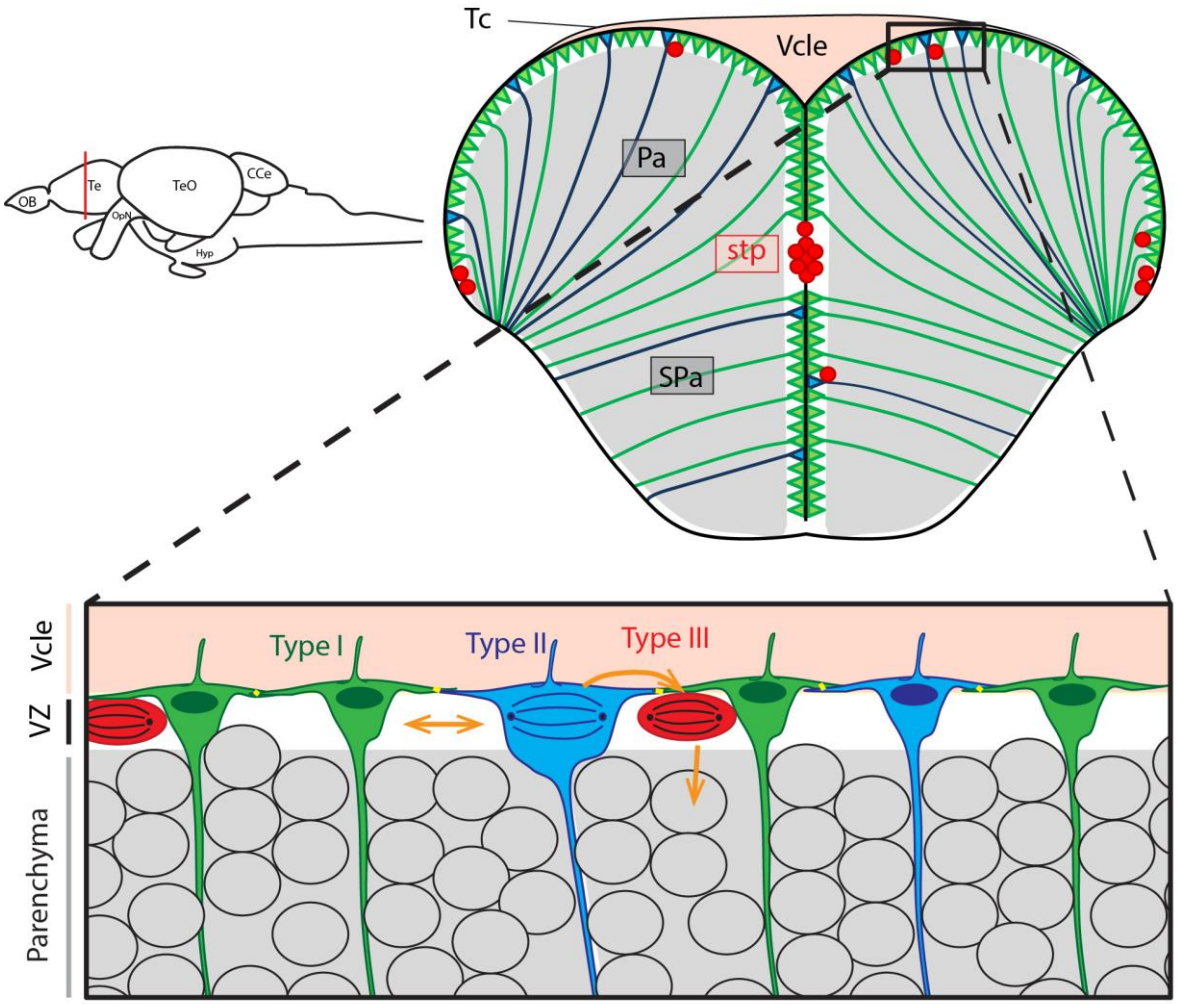


Figure 21: Schematic representation depicting the different types of progenitors of the adult zebrafish telencephalic ventricular zone (VZ)

Cross section of an adult zebrafish telencephalon at the medial level with a high magnification illustrating the organization of the ventricular zone (VZ) located at the most superficial position due to the eversion process, with quiescent RGCs (type I cells - green) that can enter the cell cycle and become proliferating RGCs (type II cells – blue). Type II cells rarely divide asymmetrically thus generating one RGC and one dividing neuroblast (type III cells – red). Type I and type II cells are found all along the ventricular zone in both the pallium (Pa) and subpallium (Spa), are linked via tight junctions (yellow dots) and directly in contact with the ventricle (Vcle). Type III cells are mainly concentrated in the RMS-like stripe (stp) that will reach the bulb and generates olfactory neurons, but they are also found along the VZ and generate the telencephalic neurons of the parenchyma (grey).

3 Signals controlling the maintenance and/or recruitment of neural progenitors/stem cells

Brain formation relies on multiple steps orchestrated via intrinsic and extrinsic signals that allow the generation of highly diverse differentiated cells while preserving pools of stem cells. Progenitor maintenance involves the maintenance of “stemness”, corresponding to the capacity of a progenitor to self renew and preserve its fate-generating properties over successive divisions, and cell survival. How “stemness” is encoded is unclear, but so called “stemness genes” include *Sox2* (Choi et al., 2014). Finally, if we consider that the progenitors possess a “cell division clock” that specify them to perform a certain number of divisions throughout life (Encinas et al., 2011), progenitor maintenance also goes through the maintenance of the appropriate rate of cell divisions as an overactivation of progenitors would causes premature depletion. Thus, the rate of proliferation could be important for progenitor maintenance, but the division mode also: as an excess of symmetric cell divisions could lead to tumorous overgrowth, whereas precocious asymmetric divisions could result in underdeveloped brain areas and, possibly, low regenerative capacites. The control of progenitor maintenance and recruitment is exerted both on embryonic and adult neural progenitors. The involved signals are produced locally by the stem cells themselves or by the stem cells niche, they could also come from long-distance sources and reach stem cells by the blood circulation or the cerebro-spinal fluid (CSF) or distant cell-cell contacts. In this third section, we will focus first on the signals involved in controlling the proliferation and maintenance of the different embryonic neural progenitors that compose the developing central nervous system, and then on the activation and maintenance of the adult neural progenitors/stem cells.

3.1 Proliferation and maintenance of neural progenitors during development

Embryonic neural progenitors are submitted to multiple signals that regulate the patterning of the developing neural sheet (see section 1.2). In addition to patterning, these pathways directly or indirectly impact neural progenitor properties such as maintenance or/and proliferation.

Notably, they confer to particular subdomains a neurogenic activity while complementary regions display delayed neurogenesis. Thus, different modes of embryonic neural progenitors maintenance and neurogenic activity co-exist within the neural plate and lead to the existence, at a given time point, of at least two types of embryonic neural progenitors. I

will first discuss how actively neurogenic embryonic neural progenitors are induced and maintained by Notch signals during early development, while in other regions progenitors are spared for later neurogenesis events. And finally, I will introduce the other signaling pathways that can be involved in influencing the maintenance and the balance between proliferation/differentiation of the embryonic neural progenitors.

3.1.1 Identification of competent proneural domains within the anterior neural plate

Neural plate induction in ectodermal cells during gastrulation (see section 1.2) is followed by the commitment of embryonic neural progenitors to a neural fate and this does not occur homogenously and simultaneously throughout the neural plate.

In Drosophila, neurogenesis first starts at particular sites and this neurogenic pattern is induced by positional cues conferring “differentiation competency” to some embryonic neural progenitors. Proneural genes from the *acheate-scute* complex are initially expressed at low level in the future neurogenic field (“proneural cluster” cells) making it competent for neurogenesis. These proneural genes then drive expression of DSL (Delta/Serrate/Lag2) Notch ligands in the entire proneural sheet (Cau and Blader, 2009).

Similar mechanisms seem to be at play in vertebrates where “prepattern” genes define competent fields of neurogenesis and activate proneural factors. The latter include orthologues of the *achate-scute* bHLH gene family (*Ash, Ato/Neurogenin*).

Studies in zebrafish and Xenopus have considerably increased our understanding of how primary neural induction and neurogenesis patterning are linked. Concerning the downstream targets responsible for neural specification, a cascade of transcription factors are induced and act synergistically with the different signaling pathways to specify neural fate. As we have seen, neural fate acquisition requires inhibition of BMP function from the dorsal neuroectoderm, and this downregulation goes first through Wnt signaling which activates repressors belonging to the *Iroquois* (*iro/irx*) genes family such as *Xiro1* in Xenopus (Gómez-Skarmeta and Modolell, 2002) and *iro3* in the zebrafish (Kudoh and Dawid, 2001). The *Iroquois* genes are then involved in activating proneural genes, as demonstrated for *iro7* in the zebrafish embryonic hindbrain (Lecaudey et al., 2004).

Second, The *Sry-related HMG* genes belonging to the *SoxB1* familly, such as *SoxD* and *Sox2*, and the zinc finger factor gene *Zic1*, are directly induced by the BMP inhibitor Chordin (Mizuseki et al., 1998a, 1998b). *Sox2* is responsible for making ectodermal cells competent to respond to extracellular signals and acts synergically with FGF signaling to initiate neural induction (Mizuseki et al., 1998a). *Zic1* is responsible for indirectly inducing *neurogenin1 (neurog1*) expression, one of the main proneural genes, either via *SoxD* expression -the

latter being sufficient by itself to ectopically induce neural tissue- (Mizuseki et al., 1998b), or, together with *Zic3*, via the activation of *POU2,* aPOU-domain family gene (Matsuo-Takasaki et al., 1999). Later, *SoxB1* genes are involved in maintaining neural progenitors as shown in the chick, where they inhibit neurogenesis (Holmberg et al., 2008).

As mentioned, the expression of prepattern genes results in the activation of proneural genes that define proneural domains, also called “proneural clusters” in the zebrafish developing CNS and “compartment” regions in the mammalian embryonic brain. The proneural genes have several functions in the neural plate. First, they have the ability to initiate Notch signaling via their positive action on the expression of the Notch ligand Delta. Notch activation is then involved in maintaining neural progenitors within the neurogenic domains (see section 3.1.2). Interestingly, there is also evidence that some proneural genes such as *Mash1* (*Ascl1*) can promote proliferation during telencephalon development via activating expression of several cell cycle genes (Castro et al., 2011). Proneural genes are also involved in specifying different neuronal cell types during development. For example, *Neurogenin1/2 (Ngn1/2*) is expressed in the dorsal telencephalon and necessary and sufficient to generate glutamatergic neurons, whereas *Mash1* is expressed in the ventral telencephalon and leads to GABAergic interneurons production (Wilkinson et al., 2013). These factors are thus essential during CNS development and have key roles regulating the balance between proliferation and differentiation of the neural progenitors.

Complementary to the “proneural clusters”, embryonic non-neurogenic domains defined as “progenitor pools” in the zebrafish or “boundary” region in the mouse do not express proneural genes but express different sets of transcription factors. These domains generally correspond to signaling centers, such as the Zona limitans intrathalamica (ZLI) or the mid-brain hindbrain boundary (MHB) (Stigloher et al., 2008) (details about this particular subtype of embryonic neural progenitors will be developed in section 3.1.3).

3.1.2 The Notch signaling pathway as the main actor in embryonic progenitor maintenance

After proneural clusters induction, the selection of commited neural progenitors is triggered by the emergence of the Notch-mediated “lateral inhibition” process. After its initiation at neural plate stage, this pathway will be one of the major signals controling neural progenitor/stem cells maintenance and differentiation. It acts locally through cell-cell interaction and has been first discovered in the fruit fly by analyzing a mutant with a serrated wing margin phenotype (Dexter JS 1914). The subsequent cloning of *Notch* identified it as a receptor interacting with two ligands in fly, Delta and Serrate (Artavanis-Tsakonas et al., 1983; Fleming et al., 1990; Vässin and Campos-Ortega, 1987). In the next part, we will

expose how the Notch pathway functions and discuss its implication on neurogenesis via the lateral inhibition process and asymmetric cell divisions, focusing mainly on vertebrates.

3.1.2.1 The mechanisms and components of Notch signalling

In mammals, the four Notch receptors (NOTCH 1-4) are type I transmembrane proteins composed of (i) an extracellular domain (NECD) containing around 30 epidermal growth factor (EGF)-like repeats, three LIN repeats and a heterodimerization region; (ii) a transmembrane domain; and (iii) an intracellular portion containing a RAM domain, six ankyrin repeats, a transactivation domain, and a carboxy-terminal PEST sequence (Figure 22) (Kovall and Blacklow, 2010). As in Drosophila, Notch interacts with ligands of the DSL (Delta/Serrate/Lag-2) family, which are type I transmembrane proteins as well (Figure 22). Five DSL ligands are separated into two subgroups: Delta-like (DLL1,DLL3, DLL4) and Serrate-like (JAGGED1 and JAGGED2). All Notch ligands contain an amino-terminal domain and several EGF-like repeats, and, in addition, JAGGED ligands contain a cyctein-rich domain. DLL3 is the most divergent and functions as a Notch antagonist (Ladi et al., 2005). Glycolsylations of the NECD and protease–mediated cleavages are involved in the maturation of the Notch receptor and are thus essential for the activity of the pathway (Noah and Shroyer, 2013).

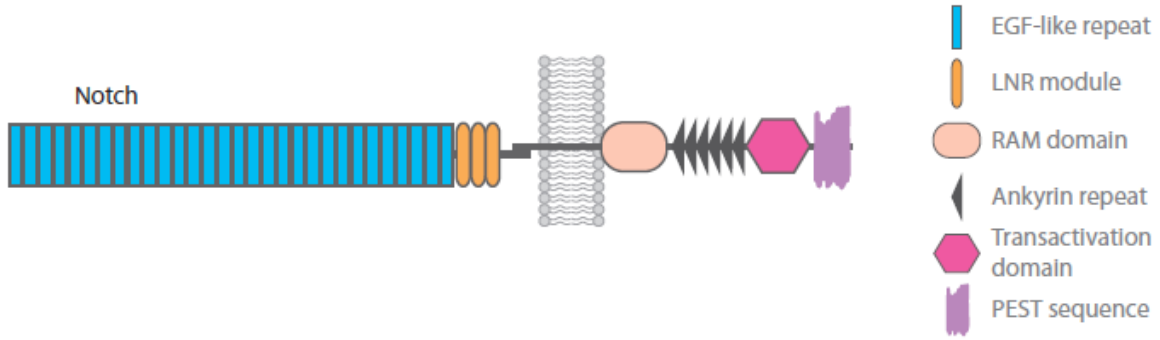


Figure 22: Structure of the Notch receptor

Each component that composes the receptors and ligands is indicated.(Noah and Shroyer, 2013)

The mature Notch receptor is translocated to the plasma membrane of the signal-receiving cell and its activation occurs by binding of DSL ligands present on the membrane of adjacent cells, the signal-sending cells (Figure 23). Upon activation, proteolysis of the receptor is carried out by a metalloprotease of the ADAM family (van Tetering et al., 2009) and the γ-secretase enzymatic complex leading to a release of the intracellular part of the Notch receptor (NICD) into the cytoplasm. Whether this cleavage occurs at the membrane or after endocytosis of the receptor is still unclear. The NICD is then translocated into the nucleus and binds, via its RAM and ankryin domains, to the DNA-binding transcription factor CSL (CBF-1/RBP-Jκ, Su(H), Lag-1). This process converts the CSL complex from a repressive to an active form by displacing corepressors and recruiting coactivators, such as mastermind-like protein (MAML1/2/3). These events lead to the transcription of target genes such as the *Hairy* and *Enhancer of split* related genes (*Hes*/*Hey*), and particularly *Hes1* and *Hes5* (Noah and Shroyer, 2013). Endocytosis is a key regulator of Notch signaling, at the level of both DSL ligands, which are endocytosed into the signal-sending cell together with the cleaved NECD, and Notch receptor, as inactivated Notch receptors can be endocytosed and recycled back to the membrane (Noah and Shroyer, 2013). Endocytosis, via ubiquitination of the ligands and receptors, is thus one way in which cells can control Notch activity. In addition to this classical view of the pathway, the existence of “non-canonical” Notch pathways has been demonstrated. Two of them do not act through the CSL complex; in the first one, Notch is cleaved but the transcription is not activated by the CSL complex (Type I), and in the second one, Notch receptor is not submitted to cleavage and does not act via CSL (Type II). Moreover, a third “non-canonical” pathway has been shown to act without Notch cleavage and through the CSL complex (Type III), indicating that the CSL complex could also integrate signals from other pathways (Sanalkumar et al., 2010a).

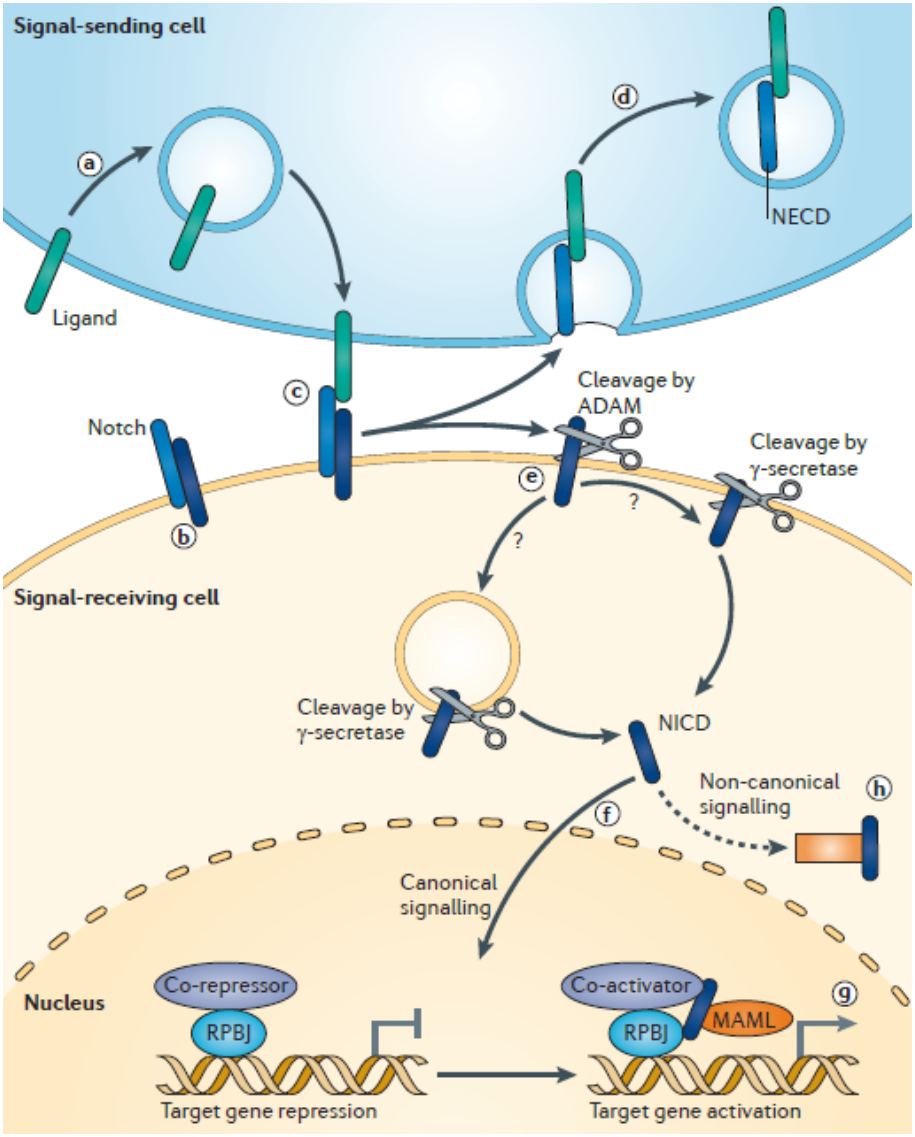


Figure 23: Notch signal transduction

In Notch signaling, a 'signal-sending cell' presents the Notch ligand (the 'signal') to the 'signal-receiving cell', which expresses the Notch receptor. Notch ligands, such as DSL (shown in green), are presented on the membrane and subsequently endocytosed (a). Notch forms a heterodimer (shown in dark and light blue) that is presented on the cell membrane (b) and upon the binding of DSL on Notch receptor (c), the Notch heterodimer is pulled apart through the force of endocytosis in the signal-sending cell, thereby trans-endocytosing the Notch extracellular domain (NECD) (d). The Notch domain that remains on the signal-receiving cell is cleaved by metalloprotease domain-containing protein (ADAM) and subsequently by γ-secretase. The precise location of the γ-secretase cleavage is controversial, with some data indicating that it occurs in the endosome and other data indicating that it can happen both on the membrane and in the endosome, leading to different Notch intracellular domain (NICD) molecules (e). In either case, after cleavage, NICD translocates to the nucleus in which it binds to the CSL, which includes RBPj, and replaces the corepressor complex by coactivators including Mastermind (g). More recently, it has been determined that Notch signalling can occur in the absence of transcriptional activation, through protein–protein interactions, or that it can activate non-RBPJ-dependent transcription (not shown), collectively referred to as 'non-canonical' signaling (h). Adapted from Ables et al., 2010.

3.1.2.2 The Hes/her genes

In the central nervous system, both canonical and non-canonical Notch signaling pathways lead to the activation of *Hes/her* genes expression (Noah and Shroyer, 2013; Sanalkumar et al., 2010a). In mammals, Hes proteins are orthologs of the Drosophila Hairy and Enhancer of split factors, which negatively regulate neurogenesis, notably through antagonizing proneural genes such as *Achaete-scute* complex (Akazawa et al., 1992; Sasai et al., 1992). In Drosophila, Enhancer of split *(E(Spl))* genes, but not *Hairy* genes, are Notch targets (Fischer and Gessler, 2007). In contrast, in vertebrates, *Hes* genes expression can depend or not on Notch but this is not related to their proximity of sequence with either *Hairy* or *Enhancer of split.* Hes proteins belong to the bHLH (basic Helix-loop-Helix) - Orange family that comprises also the Hey, Helt and Stra13/Dec subgroups (Sun et al., 2007). In mammals, there are seven protein members in the Hes family (Hes1-7), except in the mouse where no *Hes4* gene has been reported (M. Coolen, personal communication). Each *Hes* gene encodes a conserved bHLH domain, serving to form dimers through its HLH region and to bind DNA at the “N box” (CACNAG) and “class C” sites (CACG(C/A)G) through its basic domain (b) (Figure 24) (Kageyama et al., 2008). The second domain common to all Hes proteins is the Orange domain, likely involved in protein-protein interactions (Dawson et al., 1995). In C-terminal, Hes protein all possess a WRPW (Trp-Arg-Pro-Trp) motif which functions as a repressor domain via recruiting co-repressors, such as Groucho homologs (Figure 24) (Fisher et al., 1996; Grbavec and Stifani, 1996). The region between the Orange and the WRPW domains is a proline-rich domain, the size of which diverges depending on the Hes protein (Figure 24) (Sun et al., 2007). The Hes proteins are thus transcriptional repressors that inhibit their target genes by binding to their promoters and recruiting co-repressors. In addition to this active repression, Hes proteins can also inhibit transcription by forming heterodimers with activator-type bHLH factors such as E-proteins (E47 for instance) or bHLH activators such as Mash1, responsible for activating proneural target genes via its binding on E-box present in the promoters. These heterodimers do not bind to the DNA; thus, Hes factors inhibit activation of the target genes expression by sequestering transcriptional activators (Kageyama et al., 2008).

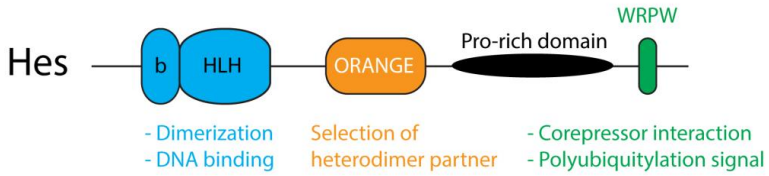


Figure 24: Structure of Hes proteins

Hes factors have a conserved basic helix-loop-helix (bHLH) domain in the N-terminal region and a WRPW domain at the C-terminus. Hes proteins form dimers and bind to the DNA through the bHLH domain and recruit co-repressors through the WRPW domain. Adapted from Kageyama et al., 2008

Phylogeny of Hes/her genes

Similarly to the situation in mammals, Hes-related (Her*)* proteins, orthologous to the HES factors, exist in zebrafish. Comparisons of sequences of *Hes/HES* and *her* genes between mouse/human and zebrafish performed by M.Coolen in the lab indicate that *Hes1*, *Hes3* and *Hes4* (only present in human) each possess only one orthologous gene in zebrafish, respectively *her6*, *her3* and *her9* (Figure 25A). However, *Hes2*, *Hes5*, *Hes6* and *Hes7* can have up to 9 orthologous genes present in the zebrafish genome (Figure 25A). During my PhD, I was particularly interested in the *her4*, *her6* and *her9* zebrafish genes. I will thus expose more details about their homology with mammalian *Hes* genes.

The most important difference between mouse and zebrafish regarding homology between *Hes/her* genes concerns *Hes5*. Indeed, when we analysed the location of orthologous sequences in the zebrafish genome, 9 genes were found to possess a sequence close to *Hes5: her4.1* to *her4.5, her12, her15.1, her15.2* and *her2*. Comparisons with the *Hes5* orthologous sequences in the Xenopus and chick genomes enabled us to define two categories within the mouse *Hes5* orthologous genes. First, sequences very close to mouse *Hes5* (also called *Hes5.1*) are *Hes5.1*, *Hes5.3* to *Hes5.7* in Xenopus, *Hes5.1* in chick, and *her4.1* to *her4.5* in zebrafish (Figure 25B). Second, *Hes5* orthologous genes more related to Xenopus *Hes5.2* are lost in eutherians (comprising mouse and human) but present in the genome of organisms “up” to marsupials (Figure 25B): in Xenopus, *Hes5.2* is present and is orthologous to *Hes5.2* and *Hes5.3* in the chick and to *her12*, *her15.1* and *her15.2* in the zebrafish. Finally, a last *Hes5*-orthologous gene is present in zebrafish, *her2.* However, it has extensively diverged and it is difficult to determine whether it is closer to *Hes5.1* or to *Hes5.2*. Contrary to the *Hes5* orthologous genes, only *her6.1* in the zebrafish is orthologous to the mammalian *Hes1* gene. Interestingly, even though the situation is similar to the Xenopus and chick, the zebrafish is a particular case within teleosteans as others possess *her6.1* and *her6.2* orthologous to *Hes1*.

Finally, as already mentioned, the *Hes4* gene exists in mammals but has been lost specifically in rodents as neither the rat nor the mouse genome contain any *Hes4* orthologous gene, whereas *Hes4* is found in humans (Figure 25C). As for *Hes1*, only *her9* is orthologous to *Hes4* gene in the zebrafish.

It is worth mentioning that *Hes1* and *Hes4* sequences are quite close to *hairy*, while *Hes2/3/5/6/7* resemble more *E(Spl)* genes but as previously mentioned, this is not endowing them differential Notch sensitivity (Sun et al., 2007).

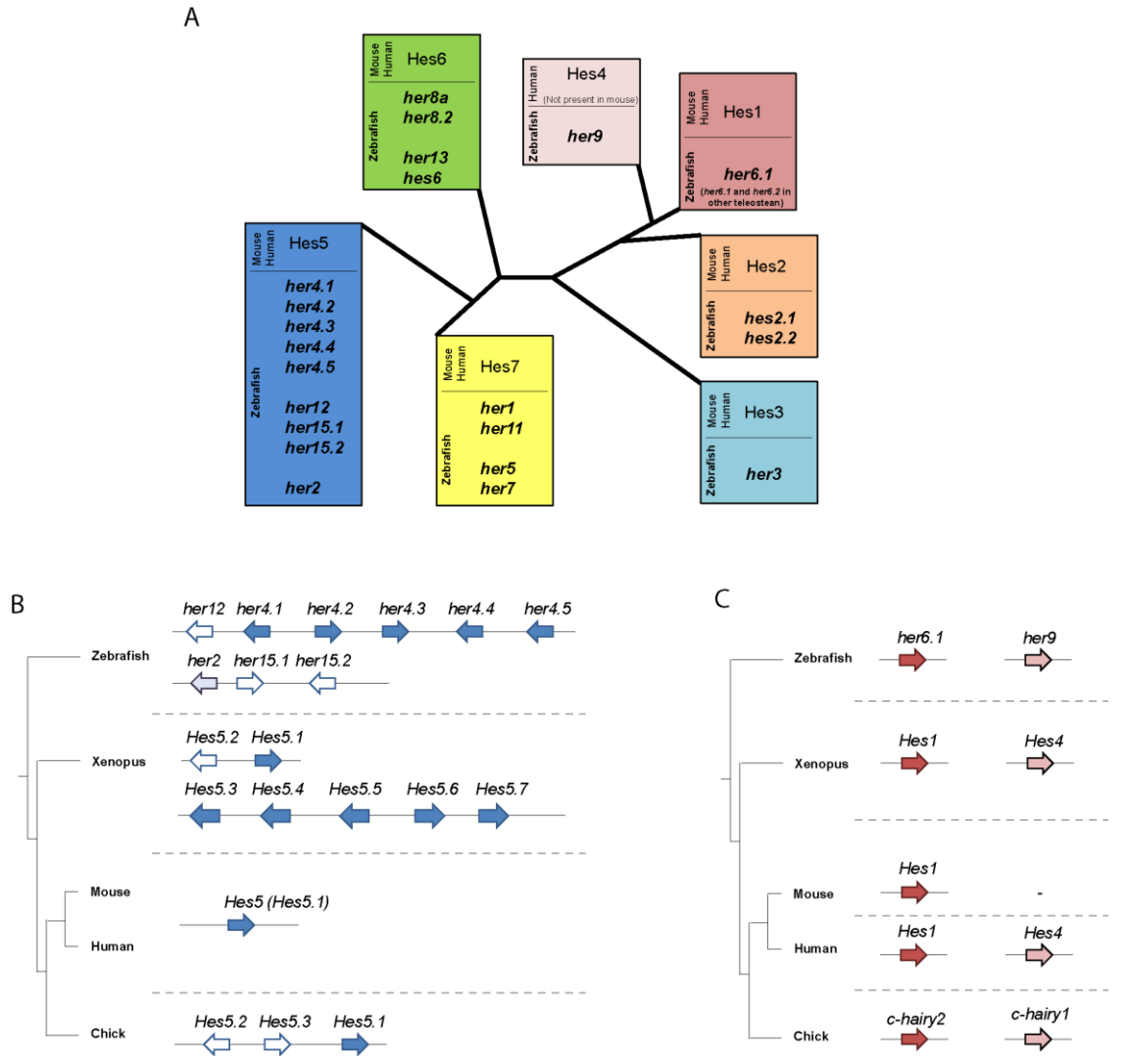


Figure 25: Phylogeny of Hes/her genes

A: General phylogeny of the different mammalian Hes genes (mouse and human) with the zebrafish her genes. B: Detailed phylogeny of the Hes5 orthologous genes between zebrafish, xenopus, chick, mouse and human with the Hes5.1- and Hes5.2-related genes labelled respectively with blue arrows or white arrows. C: Detailed phylogeny of the Hes1 (red) and Hes4 (pink) orthologous genes between zebrafish, xenopus, chick, mouse and human. Arrows represent the orientation of the sequence in the genome. (From M.Coolen – personal communication)

Function of Hes/her genes in embryonic neural progenitors in vertebrates

her genes are expressed in embryonic neural progenitors of the zebrafish. Two different sets of *her* genes are defined regarding their Notch sensitivity and characterize proneural clusters/compartment versus progenitor pools/boundary domains (Stigloher et al., 2008). The canonical *her* genes comprises *her4*, *her15 (her1.1 and her15.2)*, *her2* and *her12* and at least *her4* and *her15* are controlled by Notch signaling as both genes expression can be induced by NICD overexpression and *her15* is downregulated in the absence of Notch signaling (Bae et al., 2005; Takke et al., 1999). The neurogenic proneural clusters are characterized by canonical *her* genes expression (Stigloher et al., 2008).

On the contrary, the non-canonical *her* genes comprising *her6*, *her9*, *her5* and *her11* are expressed in progenitor pool/boundary cells in which neurogenesis is delayed. "Non-canonical" *her* genes are defined by their expression independent of Notch signaling (Stigloher et al., 2008). They are activated by positional cues, such as for *her5* in the midbrain-hindbrain boundary (Geling et al., 2003)

It is important to mention that this difference regarding canonical and non-canonical *her* genes is present in the zebrafish whereas in the mouse, the same *Hes* gene can be either Notch-dependent or Notch-independent depending on the cellular context, particularly for *Hes1* (Stigloher et al., 2008) (see section below).

In the mammalian developing central nervous system, only three *Hes* genes are expressed, from very early stages of development (E7.5). Already at the neural plate stage*, Hes1* and *Hes3* are expressed in NE cells but this expression is independent of Notch activity (Hatakeyama and Kageyama, 2006). Later, *Hes3* is downregulated and *Hes5* is expressed only in progenitors located in neurogenic regions, and this induction is correlated with the expression of Notch components such as *Notch1* and *Dll1* suggesting that *Hes5* is activated by Notch signaling (Hatakeyama and Kageyama, 2006). Contrary to *Hes3* and *Hes5, Hes1* is expressed in both neurogenic and non-neurogenic territories, including in the telencephalic region where *Hes1* is expressed at the level of the non-neurogenic presumptive cortical hem and choroid plexus domain until E11.5 (Imayoshi et al., 2008). It has been proposed that *Hes1* expression can oscillate and these oscillations are necessary for maintaining progenitors in a proliferation state (this concept will be developed in the next part – see section 3.1.2.3). This particular type of expression is thought to be typical of embryonic neurogenic regions and dependent on Notch signaling, whereas non-neurogenic progenitors display a non-oscillatory/Notch-independent *Hes1* expression (Kageyama et al., 2008; Stigloher et al., 2008).

In *Hes1;Hes5* double and triple *Hes1;Hes5;Hes3* knock-out mice, a rapid depletion of embryonic neural progenitors was observed (Hatakeyama and Kageyama, 2006; Hatakeyama et al., 2004; Imayoshi et al., 2008). Interestingly, in the telencephalon, no

severe phenotypes occured in this triple mutants, but a member of the Hey bHLH-Orange family, *Hey1*, is upregulated suggesting that it can compensate for the absence of Hes proteins (Imayoshi et al., 2008). This phenotype of progenitor depletion is also observed when inhibition of the Notch pathway is performed in *Nestin*-positive progenitors, confirming the important function of the Notch pathway on progenitor maintenance (Imayoshi et al., 2010). Although they have many targets in different contexts, in the developing brain the most important class of genes inhibited by Hes proteins are proneural genes. In the triple *Hes1;Hes5;Hes3* knock-out mice, in addition to neural progenitor depletion, up regulation of proneural genes and a premature neuronal differentiation are observed (Imayoshi et al., 2008). These results indicate that Notch signaling is necessary for maintaining the embryonic neural progenitors through its positive control on *Hes* genes expression.

3.1.2.3 Principle of lateral inhibition, oscillations and the basis of proliferation

In the compartment/proneural cluster regions, Notch pathway is active and relies on cell-cell interactions. This creates, within the neurogenic progenitor population, complementary patterns of expression of Notch receptors and their ligands. This particular type of expression is based on the mutual repression of the ligand and the Notch targets, and is called the "lateral inhibition" process. Indeed, a small bias of DSL expression in between adjacent progenitors triggers an amplifying situation that leads to an increase in Notch signaling in one progenitor compared to the other. One cell thus become Notch ON and expresses *E(Spl)* transcription factors, and the other one Notch OFF and expresses proneural genes (Skeath and Thor, 2003), the latter becoming competent to differentiate into neurons.

In mammals and zebrafish, lateral inhibition is the model that is currently proposed to explain the salt-and-pepper expression pattern of *Hes/her* and proneural genes in the neurogenic regions (Shimojo et al., 2008; Stigloher et al., 2008). In the mouse, *Dll1* and *Notch1* are induced in the neurogenic progenitors that signal to one another, generating the expression of *Hes1* in the Notch-positive progenitor. It is worth noting that one target of Hes1 is the *Hes1* gene itself. Thus, an auto-regulation of *Hes1* on its own expression would generate an oscillatory expression pattern of Hes1 in the neural progenitors (Figure 26A-B). Some evidence for this type of regulation has also been reported in mouse ES cells and fibroblasts, and with *Hes7* in the presomitic mesoderm (Harima et al., 2014). Due to this particular type of expression, proneural genes, inhibited by *Hes1*, are periodically inhibited and thus oscillate as well with a reverse period (Kageyama et al., 2008). Finally, Dll expression oscillates due to the oscillations of proneural genes responsible for its transcriptional activation. Thus, in the neural sheet at time t, some cells highly express *Hes/her* genes and a low level of proneural genes and *Dll*, and the complementary cells highly express *Mash1* or

Ngn2 and *Dll*, and a low level of *Hes*/*her* genes (Figure 26 B), triggering a salt-and-pepper expression pattern. At the peak of proneural gene oscillation, cell have a higher tendency to differentiate into neurons (Figure 26A), but this tendency is not decisive and oscillations would just confer to the embryonic neural progenitors the neurogenic competency (Imayoshi and Kageyama, 2014). Some additional cues must be involved in stabilizing proneural expression and thus trigger neuronal commitment from these « neurogenic competent » progenitors.

The functional significance of these oscillations remains to be determined but both *Hes1* overexpression and knockdown inhibit proliferation of the fibroblasts (Yoshiura et al., 2007), and sustained expression of *Hes1* in neural stem cells inhibits proliferation (Imayoshi et al., 2013). This indicates that, in addition to its role in maintaining the “stemness” of the embryonic neural progenitors, Hes1 oscillations are involved in allowing the progenitors to progress into the cell cycle.

To conclude, the lateral inhibition process allows selecting, within a population composed of cells with an equivalent potential, some progenitors to engage them into commitment and produce neurons.

3.1.2.4 Notch signaling and intralineage fate

The production of neurons can also occur during an asymmetric neurogenic cell division producing one neuron (or committed precursor) and one progenitor. In this case, which reflects an “intralineage” decision, the question is how the cell generates an asymmetric fate. Two scenari can be drawn: either the fate of the two daughter cells is imposed during cell division with the asymmetric segregation of fate determinants that trigger the commitment of one of the daughters, or the fate choice is decided after cell division through signals taking place between the daughter cells themselves. Notch activity appears involved in determining intralineage asymmetric fate choice during cell division.

In zebrafish, Dong and colleagues have shown that, in the forebrain, during asymmetric neural progenitor division, the basal daughter displays high *her4* and *her15.1* expression levels suggesting an asymmetric activation of the Notch pathway in the two daughter cells, with the cell keeping progenitor features displaying the highest Notch activity level whereas the other one differentiates into neuron (Dong et al., 2012a). Interestingly, the mother cell displays a high and uniform expression of *her4* as well as of the Notch ligand genes *dla* and *dld* before and during division.

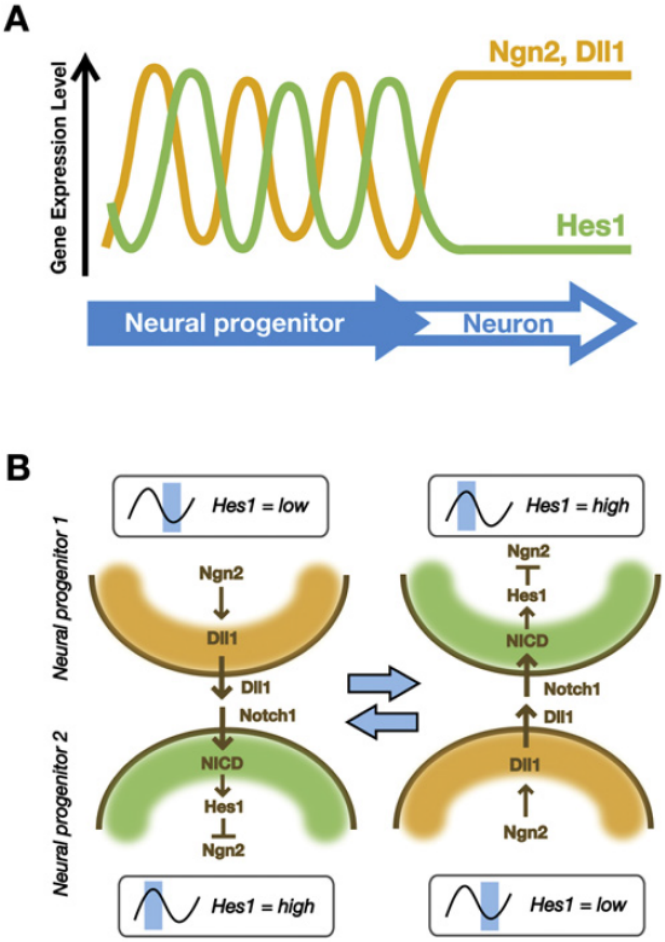


Figure 26: Model of the lateral inhibition process in mammals

(A) Expression of Hes1, Ngn2, and Dll1 oscillates in dividing neural progenitors. In immature postmitotic neurons, Hes1 is downregulated, whereas Ngn2 and Dll1 are upregulated in a sustained manner. It is likely that oscillatory expression of Ngn2 is not sufficient but sustained upregulation is required for neuronal differentiation. (B) Ngn2 and Dll1 oscillations are regulated by Hes1 oscillations in neural progenitors. Ngn2 oscillation may be advantageous for the maintenance/proliferation of neural progenitors at early stages, because it induces Dll1 expression and activates Notch signaling without promoting neuronal differentiation. From Shimojo et al., 2008

After cell division however, decreased Notch signalling occurs in the apical daugther cell in which higher *dla* and *dld* expression can be visualized. These results suggest that some event happens in the two daughter cells after division that triggers asymmetric expression of *dl* ligands and thus a differential activation of Notch signaling. These authors showed that Par3 is asymmetrically distributed and only present in the apical cell, and is involved in segregating Mindbomb (mib), a Notch promoting factor, specifically in the apical cell in order to recycle Dl at the cell surface and keep activating Notch in the basal daughter cell to maintain it as a progenitor (Dong et al., 2012a). This study showed that the main mecanism involved in asymmetric cell divisions is the segregation of the asymmetrical cell fate determinant (Par3/Mib), which can trigger a bias in the signal received by the two daughter cells and thus the differentiation of one and the maintenance of the other as a progenitor.

Interestingly, compared to the uniform expression of Notch receptors in the zebrafish neural tube, a Notch expression gradient is present in the retina within progenitors: *notch1a* RNA is enriched at the apical domain of the neuroepithelium, while *dld* and *dlc* are mostly found at the basal pole (Del Bene et al., 2008). Live imaging of *her4* expression in these embryonic retinal progenitors indicates that the Notch pathway is activated only when the nucleus moves to the apical compartement during the interkinetic nuclear migration (Del Bene et al., 2008). We can thus speculate that asymmetric cell divisions with an oblique clivage plane would generate a differential Notch activation in the two daughter cells and thus a differential cell fate. In this system, it seems that, contrary to the neural tube in which the basal daughter cell inherits the highest Notch activity level, it is the most apical cell that would keep the highest *her4* expression. Similarly, we have already mentioned that in the mammalian brain, Par3 promotes symmetric self-renewing divisions (Costa et al., 2008), indicating that depending on the regions and the species, the differential Notch activation in the two daughter cells triggering distinct cell fates is achieved by different cellular and molecular mechanisms.

3.1.3 Progenitor pools, delayed neurogenesis and non-canonical *Hes* genes

In addition to the neurogenic regions (“proneural clusters”/”compartment domains”), regions in which neurogenesis is delayed are present within the neural plate. These domains, also called “progenitor pools” in the zebrafish or ”boundary domains” in mammals, are composed of NE cells and correspond to signaling centers (Stigloher et al., 2008). They are induced in regions expressing genes inhibiting neural specification, such as *Zic2*. Zic2 acts as a Gli-antagonist, thereby limiting the neural induction-promoting effect of Shh (Brewster et al., 1998).

In the zebrafish, the second category of *her* genes, the so-called “non-canonical” *her* genes including *her3* (orthologous to mouse *Hes3*), *her9* (orthologous to human *HES4*), *her5* and

her11 (both orthologous to mouse *Hes7*), define domains in which neurogenesis is delayed (Stigloher et al., 2008). Contrary to the canonical *her* genes, experiments leading to the inhibition of the Notch pathway demonstrated that they do not require Notch signaling for their activation (Bae et al., 2005; Geling et al., 2004; Hans et al., 2004), and their expression in the neural plate is regulated by positional cues such as FGF, Wnt, or BMP signaling (Geling et al., 2003; Nguyen et al., 2000; Reifers et al., 1998). The non-canonical *her* genes are necessary to maintain the progenitors pools as their inhibition leads to up regulation of neurogenesis markers such as *neurog1*, *coe2* or canonical *her* genes, thus to their transition toward a proneural cluster (Geling et al., 2004). This transition from progenitor pool to proneural cluster-like cells likely occurs normally during development, as neurogenesis is gradually turned on in these territories over time. When I started my thesis, no progenitor pools were described in the zebrafish telencephalon.

Interestingly, the same organization has been observed in mammals. Indeed, *Hes3* is expressed exclusively in the non-neurogenic domains (Lobe, 1997), and thus could be considered as a non-canonical *Hes* gene. In addition, it is expressed prior to Notch ligands and receptors, indicating that its expression is Notch-independent (Hatakeyama and Kageyama, 2006). *Hes1*, in contrast, is expressed in both neurogenic and non-neurogenic domains, and displays a differential regulation depending on the progenitor subtypes, highlighting the different possible modes of regulation of *Hes1* by Notch signalling. Contrary to neurogenic regions where it is expressed with an oscillatory manner and under Notch control, in the non-neurogenic domains, it is expressed at a high and sustained level inhibiting neurogenesis in the domain (Baek et al., 2006). Several other signals have been implicated in the regulation of *Hes1*. Reciprocal control of *Hes1* and BMP has been suggested in the dorsal telencephalic midline (Imayoshi et al., 2008). FGF signaling has also been involved in controlling *Hes1* expression as FGF2 is able to transactivate *Hes1* via c-Jun N-terminal Kinase (JNK) pathway in neural progenitors (Sanalkumar et al., 2010b). Finally, other evidence for direct activation of *Hes1* by JNK (Curry et al., 2006), TGFα/EGF/ERK (Stockhausen et al., 2005), Shh (Ingram et al., 2008) and VEGF (Hashimoto et al., 2006) have been reported in various contexts and tissues. *Hes1* is thus more and more visualzed as a “platform” on which different signals converge and are integrated to refine the regulation of neural progenitors. However, the signals involved in the induction of the progenitor pools/boundary cells are still unclear and remain to be determined.

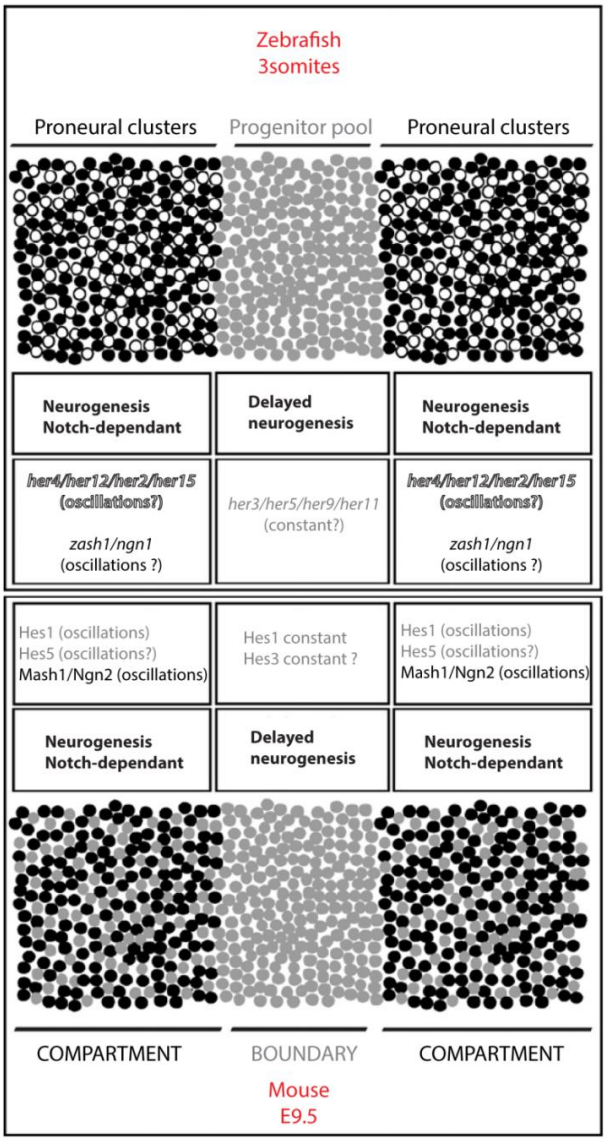


Figure 27: Comparison of the different types of embryonic neural progenitors in mouse and zebrafish

In the both zerbrafish and mouse early embryos, two types of neural progenitors are found in the developing central nervous system, the proneural cluters (also called "compartments" in the mouse) and the progenitor pools (also called "boundaries" in the mouse), that alternates whithin the neural plate.

The proneural clusters/compartments are actively involved in embryonic neurogenesis and are maintained by the Notch pathway. They display a salt and pepper expression pattern of canonical her genes (her4/her12/her15/her2 – white dotes) and proneural genes (Zash1/ngn1 – black dotes) in the zebrafish, and of Hes1/Hes5 (grey dotes) and Mash1/Ngn2 (black dotes) in the mouse. This salt and pepper expression pattern is though to reflect opposite oscillatory expression of these genes controlled by the Notch pathway as well as auto-inhibition of Hes genes on themselves; however, this type of regulation of expression was only demonstrated for Hes1 is in vitro (Harima et al., 2014).

On the contrary, progenitor pools display a delayed neurogenesis and express in the zebrafish non canonical her genes (her3/her5/her11/her9 – grey dotes), expression of which is not dependent on the Notch pathway. In the mouse, boundaries cells display a constant Hes1 expression, and Hes3 (grey dotes), type of expression of which may be constant. Adapted from Stigloher et al., 2008

3.1.4 Other signaling pathways involved in controlling the maintenance and proliferation/differentiation of embryonic neural progenitors

In addition to the Notch signaling pathway, several other signaling pathways are present in cortical progenitors and can impact progenitor maintenance, either upstream, downstream or in parallel to Notch activity. In the first part of this introduction, we have exposed some cascades of pathways responsible for specifying the anterior neural plate and the telencephalon such as Wnt, BMP, FGF and Shh. Here, we review some of their effect on embryonic neural progenitor maintenance and proliferation and differentiation. It is worth mentioning that in the embryo, all neural progenitors proliferate, unlike aNSCs that are mostly quiescent (see section 3.2).

During telencephalon development, BMP is mainly involved in the formation of the dorsal telencephalic midline as it is expressed in the roof plate and specifies the choroid plexus domain. Several BMP members are expressed in the developing telencephalon including BMP2, BMP4, BMP5, BMP6 and BMP7 (Furuta et al., 1997). However, the analysis of mutants for these genes did not provide much information on their role in neurogenesis, since some mutants die rapidly or display no obvious neural phenotypes, possibly due to redundant gene functions. In both *in vivo* and *in vitro* analyses, a first function of BMP has emerged. High BMP signalling is associated with an increase in apoptosis, in both NE cells and neurons (Furuta et al., 1997; Mabie et al., 1999). This is also emphasized by recent experiments in which electroporation of BMP7 in the medial cortical wall at E13.5 produces many cell debris in the ventricle (Choe et al., 2013), reflecting cell death of ventricle-bording cells. Thus, BMP seems to inhibit neural progenitor survival in the telencephalon. Moreover, analysis of the cell proliferation index at E10.5 reveals a low proliferation level in the dorsal telencephalic midline, indicating that BMP signaling is associated with a low proliferation rate in the progenitors (Furuta et al., 1997). In addition, culture explants of cortical primordia in the presence of BMP4 or BMP2 display a reduced proliferation of the cortical neural progenitors (Furuta et al., 1997; Mabie et al., 1999), thus indicating that, in addition to its patterning activity, BMP would impart a low proliferation rate to embryonic neural progenitors. This model holds also for the dorsal midline of the spinal cord: treatment with BMP2 on spinal cord embryonic progenitors triggers differentiation rather than proliferation of the neural progenitors (Ille et al., 2007). This highlights that BMP acts in favor of progenitor differentiation but, in certain contexts, can also regulate progenitors apoptosis, reflecting the potential heterogeneity of progenitors regarding BMP effects.

Interestingly, this study also reveals that a cross talk between BMP and Wnt signaling exists with Wnt signaling in charge of promoting proliferation in dorsal spinal cord progenitors (Ille et al., 2007). The pro-proliferative effect of Wnt signaling has been reported in many

contexts. In the embryonic brain, specific activation of the canonical Wnt pathway via stabilization of β-catenin in RGCs of at E13 leads to an increase in the cortex surface, with an over-proliferation, a disorganization of the germinal zone, and a reduction of cortical thickness with a reduced neuronal layer indicating a biais of Wnt action in favor of proliferation at the expense of differentiation (Marinaro et al., 2012). Interestingly, the RNA-binding protein Imp1 is activated by canonical Wnt signaling in fetal pallial neural progenitors and has been shown to promote neural progenitor expansion (Nishino et al., 2013). Moreover, Imp1 deficiency leads to the depletion of embryonic pallial progenitors via impairing self-renewal and triggering premature neuronal differentiation (Nishino et al., 2013). In addition, *in vitro* treatments of hippocampal neural progenitors with Wnt3a indicate that it promotes proliferation by decreasing cell cycle length without acting on differentiation, and affects neither cell survival nor the symmetric/asymmetric proportion of cell divisions (Yoshinaga et al., 2010). In addition to its role on the proliferation of ventricular progenitors, Wnt promotes intermediate progenitors (IPCs) differentiation at later stages, mainly occurring after E15.5: Wnt inhibition via electroporation of *Dkk1* at E13.5 leads to defects in neuronal production at P2 (Munji et al., 2011).
The role of Wnt signalling on zebrafish embryonic neural progenitors proliferation remains to be determined; nevertheless, it has been shown that Wnt3a and Wnt1 regulate the expansion of the zebrafish dorsal neural progenitors (Ikeya et al., 1997), suggesting a conserved function of the Wnt pathway on progenitor proliferation between mouse and zebrafish.

The other main pathway involved in the maintenance of embryonic neural progenitors is the Shh pathway. Besides its specification role in the subpallium, Shh appears involved in maintaining cortical progenitors survival: in vitro assay shoed that Shh increases cell survival, and that Shh inhibition by cyclopamine treatment leads to increased cell death (Araújo et al., 2014). Concerning its role in controlling progenitor proliferation, it has been shown in both Xenopus and zebrafish retina that Hh pathway activation increases the number of cells in proliferation, while Hh inhibition has the opposite effect. It is now suggested that this phenotype is due to modifications of the cell cycle length with an acceleration of G1 and G2 phases of the cell cycle (Agathocleous et al., 2007), which then pushes the progenitors out of the cell cycle prematurely, resulting in premature progenitor depletion. This highlights that, contrary to its protector role on cortical progenitor *in vitro*, Shh is also involved in promoting the cell cycle exit of the neural progenitors such as the retina, and thus their differentiation.

Several studies have demonstrated the implication of FGF signaling in maintaining them into a proliferative state. Indeed, FGF2 is expressed in the developing central nervous system

from neurulation stage onward in mouse (Murphy et al., 1994), and its expression, coupled with the expression of its receptors, is spatially and temporally controlled during development, concurring with neurogenesis in specific brain regions (Powell et al., 1991). Microinjection experiments and analyses of mice lacking *FGF2* have shown that it is required for cell proliferation in the cortical VZ via increasing the proportion of proliferating cells without changing the cell-cycle length (Raballo et al., 2000; Vaccarino et al., 1999). This observation has been confirmed by *in vitro* assay in which FGF2 triggers clonal expansion of neural stem cells isolated from cortical, striatal and hippocampal primordia (Rai et al., 2007; Tropepe et al., 1999). Similarly, FGF8 promotes neural progenitors self-renewal *in vitro* (Borello et al., 2008) and the reduction or overexpression of FGF8 modulates cortical size (Fukuchi-Shimogori and Grove, 2001; Storm et al., 2006). Finally, analysis of the loss of function of *FGFR1/2/3* in *Emx1*-expressing cells have confirmed that FGF signalling promotes the proliferation of cortical neural progenitors, and acts upstream of the Notch pathway (Rash et al., 2011). Interestingly, opposite functions of Fgf15/*fgf19* have been reported in mouse and zebrafish. Indeed, zebrafish *fgf19* has been shown to promote cell proliferation in the embryonic forebrain while the mouse Fgf15 inhibits proliferation (Borello et al., 2008; Miyake et al., 2005). However, they display the same inhibition effect on *Fgf8* expression in both mouse and zebrafish (Borello et al., 2008; Miyake et al., 2005), indicating that both *fgf19* and *fgf8* promote proliferation in the zebrafish but one inhibits the other. It is worth noting that at least *fgfr1-2-3* are expressed in the embryonic telencephalon of the zebrafish (Rohs et al., 2013), indicating a possible complex role of FGF signalling on zebrafish telencephalic embryonic progenitors, as already proposed in the mouse. Similarly, Rash and al also provided evidence that Fgf2 can inhibit proliferation in the hippocampal primordia by decreasing Wnt activity in the cortical hem (Rash et al., 2013); thus, it is important to keep in mind that FGF is a patterning factor and that its action can also be region-dependent.

3.2 Activation and maintenance of adult neural stem cells

Since adult neurogenesis was discovered, people tried to understand the physiological function of such a process, but also what are the signals involved in aNSCs maintenance and activation. Contrary to the embryonic context in which neural progenitors actively proliferate, aNSCs are mainly found quiescent in the adult neurogenic niches. These cells have exitted the cell cycle and are in a G0 phase, thus need to be activated to divide, in order to amplify the population but also to produce neurons.

Understanding which signals control the activation and maintenance of aNSCs can help understand many different biological phenotypes, eg. (possibly) cancer context or NSC

depletions. Moreover, the comparison of different situations, such as mammals in which aNSCs are found only in discrete forebrain regions, and the zebrafish in which they cover the entire forebrain ventricular zone, will enrich our knowledge on the control of stem cells maintenance as many signals are shared but not always used in the same way in the two species. In this section, we will introduce some pathways controlling maintenance and proliferation of aNSCs in mammals and zebrafish, with a special focus on the ones we previously discussed in the embryonic context in order to make a parallel on how the signals are used in the embryo and in the adult situations.

3.2.1 Activation of adult neural stem cells

aNSCs are mainly found quiescent. This implies that signals are involved in maintaining them in a non-dividing (or very low dividing) state. Once the stem cells are activated, they can either perform symmetric or asymmetric divisions. The balance between these two different situations results in the proper division rate and is controlled directly or indirectly by almost all the signals that are present in the body (Faigle and Song, 2013). However, it is still largely unknown how these different pathways interact with the cell cycle factors directly to make the cell reinter or stop cell divisions, how they are coordinated with one another, and whether they reflect the integration of signals coming from specific sources.

3.2.1.1 Notch3 signaling controls activation of the adult neural stem cells

Similarly to the embryonic context, *Hes5*/*her4* expression is present in aNSCs in both zebrafish and mouse and is considered as the main read out for Notch activity (Chapouton et al., 2010; Imayoshi et al., 2010; Lugert et al., 2010; Stump et al., 2002). This pathway plays a role in controlling the proliferation rate of aNSCs. Experiments leading to short term inhibitions of the Notch pathway in the adult germinal zone have shown similarities in terms of response in both mouse SEZ/SGZ and zebrafish telencephalic VZ, i.e. an increase of stem cells divisions (Breunig et al., 2007; Chapouton et al., 2010; Ehm et al., 2010; Imayoshi et al., 2010).

Interestingly, in the zebrafish, *Notch3* is expressed in the adult telencephalic VZ and the analysis of *Notch3* mutants indicates that it limits aNSC amplification by gating quiescence exit (Alunni et al., 2013). In the mouse, Notch1 is the main Notch ligand studied so far (Ables et al., 2010; Basak et al., 2012), but other Notch ligands are expressed in the germinal zones, such as *Notch3* and *Notch2* (Basak et al., 2012). It has been hypothetised that they could be responsible for controlling progenitor activation (Basak et al., 2012), but their role in aNSCs division remains to be directly determined. Interestingly, *Notch3* also limits the proliferation of satellite stem cells in the muscle (Kitamoto and Hanaoka, 2010), indicating

that the role of *Notch3* in maintaining stem cell quiescence could be a mechanism much more general than only in the brain. Nevertheless, this function of *Notch3* is not shared with high cellular turnover systems such as hematopoietic, skin epidermis or intestinal stem cells in which the Notch pathway drives different fate choices rather than controlling cell division (Perdigoto and Bardin, 2013).

In the zebrafish, several *her* genes, such as *her4*, *her6* and *her9*, are expressed in the telencephalic VZ (Chapouton et al., 2011), and short-term inhibition of the Notch pathway by pharmacological treatment of the adult zebrafish, mimicking the primary effect of *Notch3* inhibition, leads to a drastic down-regulation of *her4* expression in the entire telencephalic germinal zone, while some *her6* and *her9* expression is still present in some regions (Pers. Com. – Alessandro Alunni). This indicates that still in the adult zebrafish, *her* genes seem to be differentially regulated by the Notch pathway but whether it highlights an heterogeneity in terms of adult progenitors populations remains to be determined. In the mouse, previous investigations on the expression of *Hes* genes in the adult brain led to conflicting results. Indeed, Stump and collegues have shown an absence of *Hes1* expression in both SGZ and SEZ postnatal niches (Stump et al., 2002). In contrast, Ohtsuka and colleagues showed later that in *Hes1:GFP* mice, GFP is expressed in both SGZ and SEZ with 90% of the *Hes1*-positive cells expressing GFAP in the SGZ and only 56% in the SEZ (Ohtsuka et al., 2006).

Hes5 mRNA as well as *Hes5:gfp* expression is present in both the SGZ and SEZ (Lugert et al., 2010; Stump et al., 2002). In the SGZ, *Hes5* is expressed in both quiescent and activated "horizontal" (non-radial) stem cells, but only in quiescent radial stem cells (Lugert et al., 2010). Interestingly, these authors showed that running specifically activates the *Hes5*-positive radial stem cells whereas the horizontal (non-radial) population is not affected (Lugert et al., 2010). Finally, *Hes3* expression was not reported *in vivo* (Stump et al., 2002), while *Hes3* is expressed in *Sox2*-positive progenitors isolated from the rat SEZ (Androutsellis-Theotokis et al., 2009). Together, this indicates that, like in zebrafish, there exists an heterogeneity within the adult NSC/progenitor populations in mouse in terms of *Hes* genes expression. Whether this heterogeneity reveals a particular "state" within progenitors or highlights distinct progenitor populations remain to be comprehensively determined, and will be partially addressed in the results of this thesis' manuscript. Finally, the direct function of *Hes*/*her* genes, in particular regarding aNSC proliferation control, has not been reported so far.

3.2.1.2 Other signaling pathways are involved in maintaining the proper proliferating rate of the adult neural stem cells

Many factors participate in refining the proliferation rate and neurogenesis in adult mammalian neurogenic niches (Faigle and Song, 2013). I will only highlight here the role of signals that are also relevant for patterning, that also control embryonic progenitors, and/or that are relevant for the results I obtained during my thesis.

Similarly to the Notch pathway, Shh is involved in aNSCs activation. Smothened (Smo), one component of the Shh receptor, is expressed in the SEZ, and injection of Shh in the lateral ventricle leads to increased proliferation in the SEZ (Alvarez-Buylla and Ihrie, 2014), whereas inhibition of Shh by cyclopamine injection leads to a decrease of incorporation of the thymidine analog BrdU in this territory (Palma et al., 2005). Some type B cells and many type C cells, but not all, express *Gli1*, *Gli2* and the Shh receptor gene *Ptch1* (Palma et al., 2005). Moreover, it has been shown that Shh controls the number of neuroblasts migrating in the RMS and the migration itself (Angot et al., 2008). In the SGZ, *Ptch* and *Smo* are expressed at high levels and, similarly to the SEZ, delivery of Shh via viral infection in the dentate gyrus leads to an increase of proliferation (Lai et al., 2003; Traiffort et al., 1999). In both the SGZ and SEZ, the effect of Shh as a mitogen is confirmed by experiments in which exogenous Shh protein is added onto cultured aNSCs: in the SGZ, the cells displaying mutipotent properties expand following Shh addition (Lai et al., 2003) and, in the SEZ, Shh promotes neurosphere proliferation and differentiation in cooperation with EGF (Lai et al., 2003; Palma et al., 2005).

In both mammalian adult neurogenic niches, the Wnt pathway is active as both display Wnt signaling target expression (Adachi et al., 2007; Lie et al., 2005). In the SGZ, *Wnt3a* is expressed in both hippocampal astrocytes and adult hippocampal progenitors, the latter expressing also components of the Wnt pathway such as *Frz1* or *βcatenin* and the target genes *Axin1* and *Lef1* (Lie et al., 2005). A reduction of Wnt3a in mice leads to lower Wnt signaling levels and suppression of neurogenesis (Okamoto et al., 2011). Moreover, loss of the Wnt antagonist *Sfrp3* increases self-renewal of aNSCs and neuronal maturation in the SGZ (Jang et al., 2013), whereas loss of the other Wnt antagonist *Dkk1* activates neuronal precursors proliferation and the production of immature neurons (Seib et al., 2013), indicating a complex role of Wnt in adult hippocampal neurogenesis. In the SEZ, *in vivo* and *in vitro* experiments showed that Wnt activation promotes aNSCs proliferation (Adachi et al., 2007; Azim et al., 2014; Yu et al., 2006a). Postnatally, *Wnt3a* and *Wnt7a* are expressed in the SEZ with *Wnt3a* specifically present in the dorsal portion of the SEZ, and the choroid plexus expresses several Wnt ligand genes such as *Wnt3a, Wnt5a, Wnt7a* (Azim et al., 2014). Most cells activated by Wnt signaling express *GFAP*, but Wnt is also activated in non-*GFAP*

proliferating cells (Azim et al., 2014), and analysis of the Wnt pathway target gene *Axin2* using reporter mice indicates that it is activated in both *Mash1*-positive Type B and Type C cells (Adachi et al., 2007). Increasing Wnt signaling in the SEZ triggers an increase of Type B and Type C cells whereas no changes are visible in the number of Type A cells (Azim et al., 2014), indicating that Wnt signaling promote SEZ neurogenesis via promoting proliferation of the aNSCs and intermediate progenitors (Type C).

In the adult neurogenic regions of the mouse telencephalon, the BMP signaling pathway promotes the quiescent state of aNSCs. In the SGZ, Mira and colleagues have recently shown that BMPR-IA is expressed in aNSCs and maintains them quiescent (Mira et al., 2010). Interestingly, translocation of P-Smad1 in the BMP-induced quiescent cells *in vitro* is associated with an activation of *Hes1* expression without affecting *Hes5*, indicating that *Hes1* expression seems to be correlated with the quiescent state whereas *Hes5* is not sensitive to whether the cell is in proliferation or in quiescence. The anti-proliferative effect of BMP was already observed in cultured hippocampal NSCs treated with the BMP inhibitor Noggin (Bonaguidi et al., 2008). In addition to the expression of *BMPR-1A* in aNSCs, a scattered expression of *BMPR-II* is visible in the SGZ (Charytoniuk et al., 2000). The source of BMP signaling in this region may be the choroid plexus, which expresses *BMP6* and *BMP7* (Charytoniuk et al., 2000). In the SEZ, several BMP pathway components are expressed such as the ligands BMP2, 4, 6 and 7 (BMP4 and BMP2 being specifically expressed in endothelial cells) (Mathieu et al., 2008), the receptor BMPR-II, the transcription factor Smad4 that mediates BMP signaling, and the BMP target genes *Id1* and *Id3* (Colak et al., 2008). P-Smad1/5/8 are mainly found in the GFAP-positive cells and in a few fast-proliferating BrdU-positive cells but not in DCX-positive neuroblasts, indicating that the BMP pathway is mainly acting on aNSCs. Increased BMP signaling in the SEZ leads to a decreased number of *Nestin*-positive progenitors, suggesting a negative effect of BMP signaling on progenitor proliferation in the SEZ (Gajera et al., 2010). Similarly, NSCs originating from the SEZ cultured in the presence of BMP4 acquire cellular and transcriptional characteristics of quiescent cells via BMP4 action on NFIX factors (Martynoga et al., 2013). On the contrary, inhibition of BMP signaling by disrupting *Smad4* function in *Glast*-positive glial cells does not affect NSCs proliferation but impairs neurogenesis (Colak et al., 2008), contrasting with the results of BMP inhibition in the SGZ, and thus highlighting differences in the role of the BMP pathway on aNSCs of the two mammalian neurogenic niches.

Examples of other factors include growth factors such as FGF, IGF and VEGF. They share common principles of signal transduction with tyrosine-kinase receptors and all of them have been shown to act in favor of aNSCs proliferation and neurogenesis in both the SEZ and SGZ (Faigle and Song, 2013). Interesingly, the PDGF pathway is active in the SEZ but is

specific of oligodendrocytes production from Type B cells. Its infusion triggers massive Type B cell proliferation, one of the glioma features (Jackson et al., 2006). In terms of factors regulating quiescence, severeal recent studies have identified quiescence-promoting factors such as TGFβ1 promoting stem cells quiescence in the adult hippocampus (Kandasamy et al., 2014), but also N-cadherin protein attachement to the ependymal cells. When N-cadherin is cleaved by the metalloprotease MT5-MMP, it triggers GFAP-positive type B cells proliferation (Porlan et al., 2014). Finally, metabolic signals can also impact aNSCs proliferation such as diet (Park and Lee, 2011), physical exercise (Vivar et al., 2013) or hypoxia (Zhu et al., 2005).

3.2.2 Maintenance of adult neural stem cells

Theoretically, several processes can be involved in aNSCs maintenance, like for embryonic progenitors. However, so far, no pathways have been shown as directly involved in controlling cell survival in this context. Thus, the known pathways implicated in maintaining aNSCs mainly play a role in regulating self-renewal. Indeed, the absence of self-renewal during cell division, aNSCs will eventually generate neurons or differentiated glial cells without maintaining one progenitor, which leads to a progressive depletion of the stem cell pool. As already mentioned, overactivation of stem cells via deregulation of pathways involved in maintaining the appropriate rate of cell divisions could also be indirectly responsible for premature stem cells depletion, if stem cells in fact make a finite number of divisions. These phenomena are not always easy to discriminate and much information is necessary to fully understand the mode of action of signaling pathways on aNSCs maintenance. In this section, we report on the pathways known to regulate the “stemness” of the aNSCs.

3.2.2.1 Notch1 signaling maintains adult neural stem cells

Long-term inhibition of the Notch pathway in either *Nestin*-positive or *GLAST*-positive aNSCs indicated that Notch activity is necessary for maintaining aNSCs in both SEZ and SGZ in the mouse (Ables et al., 2010; Ehm et al., 2010; Imayoshi et al., 2010). In the zebrafish so far, no aNSCs depletion was observed upon Notch inhibition in the adult telencephalon (Alunni et al., 2013); nevertheless, it is worth noting that the inhibition experiments in the zebrafish were performed using pharmacological inhibitors disrupting γ–secretase activity and thus were not as long as, and probably as efficient as the genetic knock-outs performed in the mouse system; the level of inhibition is thus not the same in the two cases. To determine

really whether the zebrafish and mouse situations differ in terms of role of Notch in the long-term maintenance of the aNSCs, further experiments need to be performed.

However, it is interesting to note that in the adult germinal zones of both organisms, several Notch pathway components are expressed. It has been recently shown that several Notch ligands are expressed in the adult zebrafish pallial progenitors (Alunni et al., 2013; de Oliveira-Carlos et al., 2013). Notch1 is expressed specifically in proliferating progenitors, corresponding to activated aNSCs (type II) and neuroblasts (type III) (Alunni et al., 2013; de Oliveira-Carlos et al., 2013). Experiments with *notch1 vivo*-morpholino injections in the zebrafish adult telencephalic ventricle lead to a decrease in the number of activated aNSCs, indicating that Notch1 could be involved in maintaining the “stemness” of the aNSC either directly, or indirectly via permitting the completion of the cell cycle (Alunni et al., 2013). In the mouse, in vitro experiments demonstrated that Notch1 is required to maintain adult SEZ NSCs (Nyfeler et al., 2005) and long-term inhibition of *Notch1* in the *Nestin*-positive progenitors of the SEZ and the SGZ leads to a progressive depletion of quiescent aNSCs, probably due to their impossibility to self-renew during divisions (Ables et al., 2010; Basak et al., 2012). Interestingly, these results are partially similar to the ones obtained in the *RBPJ*-conditional mutant mice (Imayoshi et al., 2010), indicating that *Notch1* mainly goes through this pathway to maintain the aNSCs.

3.2.2.2 Shh signaling maintains aNSCs

The use of antimitotic molecules demonstrates that the Shh-responsive population contains slow-cycling neural stem cells that can repopulate both adult germinal zones (SEZ and SGZ) in rodents after depletion of the dividing cells (Ahn and Joyner, 2005). The Shh pathway plays an important role in maintaining NSCs of the SGZ. Indeed, analysis of conditional *Smo* mutants reveals that inhibition of *Smo* at post-natal stage (P15) leads to a drastic decrease of proliferation and a complete absence of *BLBP*-positive cells in the SGZ (Li et al., 2013; Machold et al., 2003), suggesting that Shh is involved in maintaining the stemness of the post-natal progenitors. Moreover, lineage tracing experiments indicate that aNSCs in the SGZ derive from Shh-responding cells during the late development (Ahn and Joyner, 2005), suggesting a cell-autonomous requirement for Shh signals. Finally, quantification and analysis of reporter mice for *Gli1/2/3* indicate that B1 cells in the SEZ would be the main Shh-responsive population in this region (Ahn and Joyner, 2005; Ihrie et al., 2011; Palma et al., 2005; Petrova et al., 2013). These results indicate that Shh is necessary to maintain NSCs at least from post-natal stages in the SGZ. In relation with the mitogenenic role of Shh reported previously (see section 3.2.1.2), it highlights that Shh could act on stem cells by promoting self-renewing divisions.

In terms of Shh sources, Shh protein has been detected in the dentate gyrus, the cerebrospinal fluid and the neuropil surrounding the ventral SEZ (Ihrie et al., 2011; Lai et al., 2003). In contrast to the protein localization, no clear expression of Shh has been reported in the SGZ and SEZ (Alvarez-Buylla and Ihrie, 2014). Shh-responsive cells thus integrate extrinsic signals coming from outside of the neurogenic niches, and only neurons appear to be the source for Shh, such as Neurod6-positive neurons in the SGZ (Ihrie et al., 2011; Li et al., 2013).

How Notch and Shh cooperate to regulate the maintenance of aNSCs remains unclear. In the embryo, it has been shown that both pathways can activate *Hes1* expression during cortical development (Dave et al., 2011), and *Hes* genes are responsible for maintaining embryonic neural progenitors. We already mentioned that *Hes1* is expressed in aNSCs of the SVZ and SGZ, indicating that Notch and Shh could act on *Hes* genes expression to maintain stemness of the progenitors.

4 The origin of aNSCs

What is the origin of aNSCs, and what can this origin tell us about what aNSCs can do? *In vitro* techniques based on reprogramming somatic differentiated cells to generate progenitors with various differentiation potentials (the so-called iPS technology) are more and more developed and represent a powerful system for the understanding of diseases. We already mentioned that several categories of stem cells exist depending on their differentiation capabilities, and long term stem cells must integrate processes that maintain or trigger their stemness, control their proliferation throughout life and restrict/instruct them in terms of neuronal/glial production. The cell population(s) at the origin of the stem cells, whether they are composed of differentiated cell and/or progenitors, are thus the target of numerous signals during pre-natal and post-natal development. Knowing from which embryonic populations aNSCs derive but also which signals are involved in generating the different aNSCs could help us approach the proper way to use them *in vitro,* but also *in vivo* for regenerative medicine.

4.1 Hypotheses on the origin of adult neural stem cells

Several hypotheses can explain the origin of tissue stem cells. First, these can derive from cells already engaged into the building of the organ during development. This possibility would imply that they originate from an embryonic population already actively engaged into the generation of differentiated cells at embryonic stages and that this population is maintained all along development to colonize the adult germinal niche and generate adult stem cells. Second, adult stem cells can derive from progenitors set aside in the embryo, not involved in organ differentiation at an early stage, and dedicated to the generation of adult stem cells. The embryonic population is thus maintained in a particular environment that keeps them “away” from differentiation, and colonizes the stem cells niche or becomes activated as stem cells at some point during life. Finally, a less probable hypothesis, but that cannot be excluded, would be that the adult stem cells derive from a differentiated population of cells that would have had a transient function and that would dedifferentiate or acquire stem cells properties to perform stem cells functions in the adult organ. These hypotheses are not mutually exclusive and adult stem cell populations could be heterogenous in terms of embryonic origin and thus arise from multiple sources via multiple ways.

4.2 The origin of a stem cells *in vivo*: a technological issue

Few studies have directly investigated the embryonic origin of adult neural stem cells in terms of precise cellular populations, and this may in part result from technological issues. Indeed, investigating the embryonic origin of a cell population implies the availability of long

term lineage tracing technologies that enable to permanently label a specific population of embryonic cells and follow it overtime. In the case of aNSCs, the main challenge is to manage to lineage trace embryonic cells for several weeks to months. The first lineage tracing methods developed were using viruses. These techniques were based on the capacity of viruses to infect cells and integrate their genetic material into the genome of dividing infected cells. It is thus inherited by the entire cell's progeny. For lineage tracing, viral structural genes are replaced by a bacterial *beta-galactosidase* (*Lac-Z*) gene, or another reporter gene, that allows the histochemical detection of the infected cell progeny (Sanes, 1989). This technique has been extensively used to lineage trace embryonic progenitors and get the first overview of neuronal lineages in the brain (Luskin, 1994). This technique has also been used to address the glial fate of NE cells, and the role of the Notch pathway in this lineage. Indeed, experiments using *in utero* injections of viruses modified to overexpress the intracellular domain of the *Notch1* or *Notch3* receptors in the brain ventricle of embryos at E9.5, prior to the onset of neurogenesis, demonstrate that NE cells generate radial glia when they overactivate the Notch pathway and that postnatally, these infected cells generate astrocytes and cells with aNSCs-like morphology in the lateral ventricle (Dang et al., 2006; Gaiano et al., 2000). Moreover, Merkle and colleagues showed via adenovirus injections that post-natal radial glia generate some neurogenically active and GFAP-positive cells (Merkle et al., 2004). This illustrated the lineage relationship that exists between these different cell types and the possibility that some RGCs constitute a progenitor population that will generate aNSCs.

The virus infection technique was the first possibility to perform long-term lineages and gave the first cues on aNSCs' origin; nevertheless, the main problem is that it is impossible to control precisely the region or the precise cell population that is targeted. In that respect, the genetic Cre-Lox recombination technology is more powerful. It is based on the ability of the Cre recombinase enzyme to recognize specific genetic sequences, the LoxP sites, and perform a homologous recombination between two LoxP sites leading to the excision of a particular DNA sequence and the expression of a downstream reporter. For Cre-lox lineage tracing experiments, a "driver" genetic construct carrying the coding sequence for the Cre recombinase is placed under the control of a cell type specific promoter (Figure 28A). It recombines a reporter construct in which –in most cases- a ubiquitous promoter drives expression of a floxed DNA sequence, either a STOP sequence or a reporter 1 gene, followed by another reporter 2 gene such as *GFP* or *LacZ* (Figure 28B). Upon regionalized Cre expression, the STOP/reporter 1 sequence is permanently removed and the reporter 2 gene starts to be expressed (Figure 28C). This powerful method was first available in the mouse model, then has been extended to the zebrafish (Boniface et al., 2009; Hans et al., 2011; Rodrigues et al., 2012)

However, the limitation of this approach is that it allows the visualization of the global lineage of stem cells from the onset of the stem cells gene expression, and it is not possible to discriminate between stem cells populations that would start to express at different time point the stem cells gene we choose.

This issue of temporal control was solved by fusing the Cre recombinase sequence to one or two mutated ligand-binding domains of the estrogen receptor (CreERT2): then, activation of the Cre recombinase can be achieved via treatment or injection of tamoxifen, an oestrogen analog. Compared to the previous method, it is thus possible to long-term lineage trace a precise population of cells expressing at one time point a particular gene, allowing investigations on the potential heterogenous contribution of embryonic progenitors to the formation of the aNSCs.

The CreERT2 system has been used so far to study the contribution of a whole cell population to the generation of adult stem cells. The only way of appreciating the contribution of one particular cell to a process was to perform clonal analysis based on low rate of recombination events using a very low concentration of tamoxifen. Recently, the clonal analysis systems were improved with a new technique based on the CreERT2 system, the brainbow technology (Livet et al., 2007). Brainbow was set up in order to be able to lineage trace at the same time several cells and evaluate the contribution of each of them to a process, even though these cells are closed to each other.

Compared to the classical reporter construct (Figure 28B), the Brainbow reporter transgene contains up to four different reporters flanked by different mutated loxP-like sites (lox N, lox 2272) that randomly recombine only with the identical lox sequence. Combined with a CreERT2 driver and tamoxifen treatment, the different transgene copies inserted into the genome will randomly recombine triggering the expression of only one particular reporter combination in each cell. In the case of the investigation of stem cell origin, Brainbow constitutes a very powerfull system to investigate the contribution of each embryonic progenitor to the whole aNSC population. This technique is currently avalaible in the zebrafish model (zebrabow) (Pan et al., 2013a)

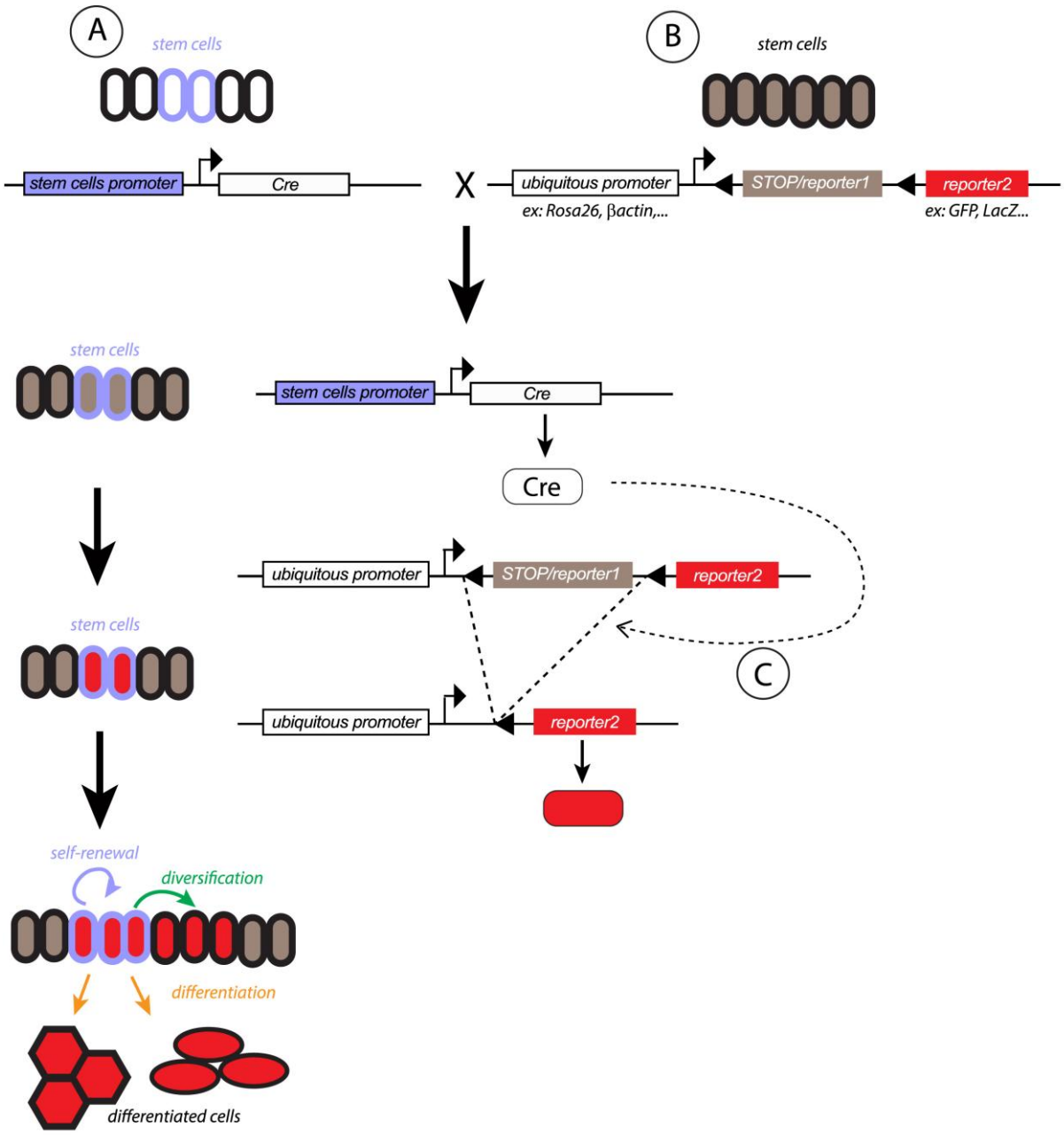


Figure 28: Model of lineage tracing experiment of an adult tissue stem cells population

See section 4.2 for detail on (A), (B), (C)

4.3 State of art on the embryonic origin of aNSCs in the mouse

In the case of the mouse brain, several studies using Cre-Lox tracing investigated the relationship between RGCs and the aNSCs, or between embryonic and adult territories, and brought complementary but still incomplete information on the origin of aNSCs; these results are summarized in Table 1.

A few studies investigated the lineage of telencephalic embryonic RGCs using either the human *GFAP* promoter or the *BLBP* promoter driving non-conditional Cre expression to lineage trace specifically RGCs. These studies showed that aNSCs of the SEZ and SGZ are positive for the reporter, indicating that they derive from GFAP-positive and BLBP-positive cells (Anthony et al., 2004; Malatesta et al., 2003). However, the limitation of this approach is that it allows the visualization of the global lineage of glial cells and it is not possible to temporally control the recombination events; in addition to this, it is known that aNSCs express also *GFAP* and *BLBP* (see section 2.2), thus the promoter could be activated at adult stage and target the aNSCs eventhough they would not derive from RGCs. These experiments were thus not sufficient to confirm that aNSCs derive from RGCs.

As mentioned previously, embryonic RGCs are submitted to different signals depending on their location during development, and it remains to be determined whether these different RGCs are heterogenous regarding their contribution to the adult stem cells niches. Ahn and Joyner took advantage of the CreERT2 approach to address the role of Shh in the control neural progenitors by lineage tracing the Shh-responding cells using a *Gli1:CreERT2* mouse line. They showed that aNSCs of the SEZ and the SGZ derive from distinct late embryonic cells that respond to Shh respectively at least from E15.5 and E17.5; in this respect, it is worth noting that the different timing of Shh activation in the precursors of the two adult germinal zones suggests that the SEZ and then the SGZ are established sequentially during mouse development (Ahn and Joyner, 2005). Concerning the source of Shh-responding cells at the origin of the neurogenic niches, a recent study has investigated this question in the SGZ and has found that the ventral part of the developing hippocampus expresses *Gli1* at E17.5. Analyses at several time points of the lineage of *Gli1*-positive cells at E17.5 indicate that at birth some of these Shh-responding cells of the ventral hippocampus migrate toward the dorsal hippocampal side and participate in SGZ generation (Li et al., 2013). However, a clear characterization of the embryonic cell population of the ventral hippocampus remains to be done. Moreover, these authors report that this population is different from the dentate neuroepithelium, composed of proliferating radial glia in the cortical part abutting the hem, and is considered so far as the population at the origin of the hippocampus. Finally, in addition to the ventrally-derived Shh signaling active cells, *de novo* Shh-responding cells are gradually produced in the developing dentate gyrus (Li et al., 2013). All these results suggest

that the aNSCs of the SGZ are heterogenous and might be derived from at least several embryonic cell populations that become Shh-sensitive at different time points during DG formation. In addition to this, *Gli1* is expressed in some ventricular domains of the developing cortex and striatum, indicating that the adult neurogenic zone could derive also from these regions (Dahmane et al., 2001). Recently, Bowman and colleagues have investigated the contribution of Wnt signaling to the formation of both the SEZ and SGZ using an *Axin2:CreERT2* line. These studies revealed that in both regions, some GFAP-positive cells involved in adult neurogenesis originate from Wnt signaling-positive cells at different stages in the embryo, from very early to post-natal stages, and demonstrated that some long-term positive progenitors persist in the adult neurgogenic regions of the mouse brain (Bowman et al., 2013). However, this study does not investigate the process at the population level, given that only mosaic recombinations were conducted, and no precise characterization of the Wnt-positive embryonic populations were performed, with respect to the different progenitor populations that compose the embryonic brain.

The contribution of regionally different progenitors to SEZ formation has been investigated using promoters of regional marker genes driving Cre expression in different ventricular subdomains, such as *Emx1* in the cortex, *Dbx1* in the pallium/subpallium boundary, *Gsh2* in the lateral ganglionic eminence and *Nkx2.1* in the medial ganglionic eminence. These lineage tracing experiments are mainly based on a non-conditional Cre/lox system coupled with *in vitro* assays. They showed that all the subdomains delimited by these transcription factors during development contribute to the formation of the adult SEZ in a regionalized manner. Interestingly, aNSCs generating the RMS migrating neuroblasts are mainly composed of cells deriving from *Emx1*- and *Gsh2*-positive domains, while *Dbx1* progenitors generate a portion of the SEZ that does not contribute to the generation of interneurons; they might play another function such as gliogenesis (Willaime-Morawek et al., 2006; Young et al., 2007). The partial cortical origin of the SEZ has been confirmed by lineage tracing experiments using Ad5-CMV-Cre-GFP (Adeno-Cre) showing that dorsal RGCs at birth generate later olfactory interneurons (Ventura and Goldman, 2007).

Lineage traced population	Technics	Results	Reference
Glial cells	*BLBP-Cre;R26R/hGFAP-Cre;R26R*	Glial cells generate the SEZ and SGZ progenitors	*(Anthony et al., 2004; Malatesta et al., 2003)*
Shh-responding cells	*Gli1-CreERT2;Rosa-CAG-LSL-tdTomato-WPRE*	Some SEZ adult neural stem cells derive from cells starting to respond to Shh at E15.5 and some SGZ adult neural stem cells derive from cells starting to respond to Shh at E17.5, that migrate from the ventral developing hippocampus to participate to the dentate gyrus formation.	*(Ahn and Joyner, 2005; Li et al., 2013)*
Wnt-responding cells	*Axin2-CreERT2;Rosa26-mT/mG*	Adult neural stem cells located in the dorso-medial portion or the SEZ and the vast majority of SGZ adult neural stem cells derive from cells responding to Wnt pathway at E12.5	*(Bowman et al., 2013)*
Cortical cells	*Emx1-Cre;R26-GFP/Emx1-CreERT2;R26-GFP/Emx1IRES-Cre;Z/EG*	Dorsal SEZ neural stem cells derive from cells Emx1-positive at E10.5	*(Young et al., 2007; Willaime-Morawek et al., 2006)*
Cells at the pallium/subpallium boundary (PSB)	*Dbx1-Cre;R26-GFP*	Lateral SEZ neural stem cells derive from Gsh2-positive cells	
Subpallial cells	*Gsh2-Cre;R26-GFP*	Dorso-lateral SEZ neural stem cells derive from Dbx1-positive cells	
MGE subpallial cells	*Nkx2.1-Cre;R26-GFP*	Ventro-lateral SEZ neural stem cells derive from Nkx2.1-positive cells	
Cortical cells	*Adeno-Cre;R26R-YFP*	Dorsal RGCs at P1/2 generate cells participating to the RMS interneurons	*(Ventura and Goldman, 2007)*

Table 1: Summary of current knowledge on aNSCs origin in the mouse brain, based on Cre/lox lineage tracing experiments

Overall, these results testing the embryonic origin of aNSCs suggest that aNSCs generation is a complex process that involves several embryonic progenitor populations. However, the precise embryonic population at the origin of the aNSCs, the dynamic of its proliferative and neurogenic state, and the pathway that are involved in controlling changes in their maintenance and activation all along development, remain to be determined. Our knowledge on the history of an aNSCs-generating embryonic progenitor would help us attain a better understanding of the different states and steps through which a progenitor must have gone to generate aNSCs.

5 Aims of the study

The first aim of this study was to determine the embryonic cell populations at the origin of the aNSCs located in the zebrafish pallium by addressing the contribution of both actively neurogenic and non-neurogenic embryonic progenitor subtypes. In order to get an overview of the process, I took advantage of several lineage tracing methods using a tamoxifen inducible form of the Cre recombinase driven either in a particular embryonic progenitor population, or in a mosaic way allowing a clonal analysis based on the use of a caged version of the tamoxifen and the brainbow system. This allowed me to collect information on the embryonic populations generating the entire pallial ventricular zone: the number of progenitors composing these populations, the cell types, and finally the dynamics of proliferation and differentiation of the different embryonic progenitors subtypes. Then, I investigated the role of some signaling pathways, especially Notch, in controlling the maintenance and the activation of the embryonic and juveniles progenitors in order to understand the signals responsible for the emergence of aNSCs. Finally, in addition to the interest of the study regarding the maturation of the progenitors during development, it brought up new information on the construction of the zebrafish pallium, highlighting its different compartments and their homologies with the mouse telencephalon.

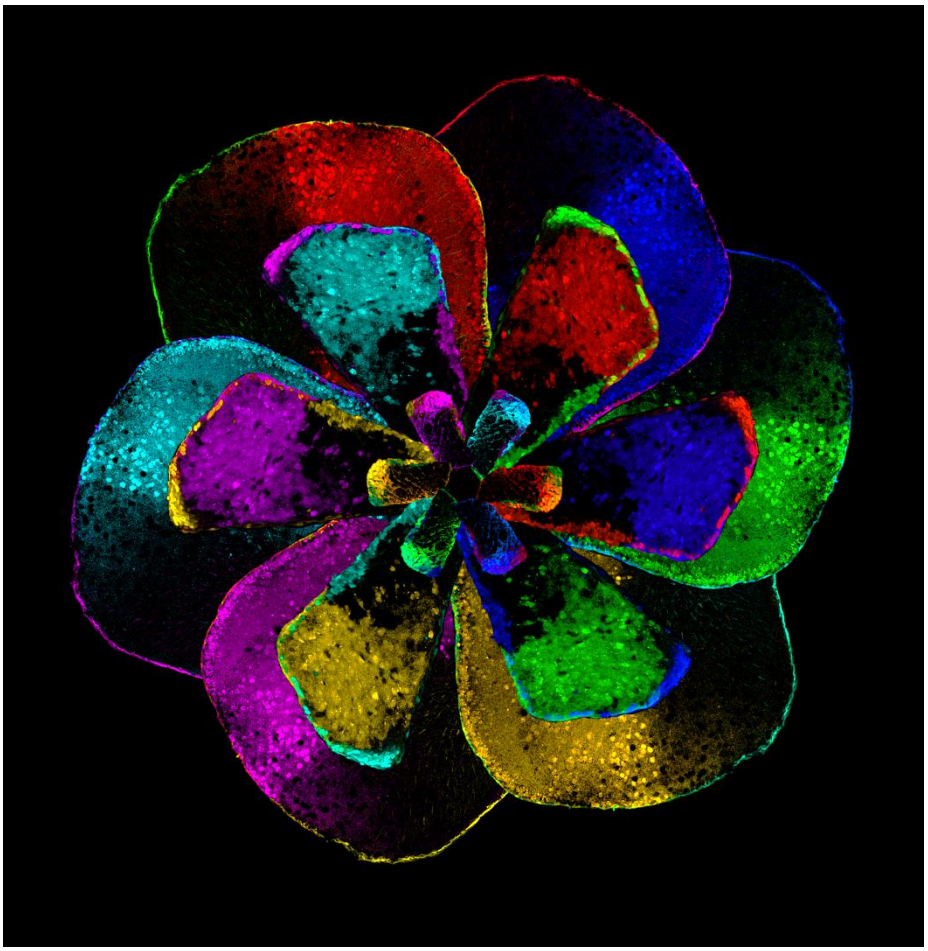
Specific aims:

1. Analyze the progeny of the *her4*-positive, actively neurogenic embryonic progenitors, in terms of aNSC generation in the pallium
2. Analyze the contribution of non-neurogenic progenitor pools to aNSC generation in the pallium
3. Get an overview of the dynamics of growth of the different pallial domains along development
4. Determine the role of Notch signalling in maintaining the different types of embryonic and juveniles neural progenitors along life
5. Determine the homologies between the compartments of the zebrafish and the mouse pallium by analyzing expression of specific markers
6. Appreciate the organization and the timing of formation of the different neuronal pallial compartments

CHAPTER II: RESULTS

CHAPTER II: RESULTS

1 Embryonic origin of adult pallial neural stem cells



1.1 Specific aims and main results

aNSCs are found in the telencephalon of many vertebrates such as the mouse, the chick, and the zebrafish (Chapouton et al., 2007). They are characterized by self-renewing capacity and multipotency, and are involved in producing adult-born neurons (Kriegstein and Alvarez-Buylla, 2009). In the mouse field, they are considered as emerging from radial glial cells (RGCs), embryonic neural progenitors in charge of brain development (Anthony et al., 2004; Malatesta et al., 2003), themselves deriving from NE cells composing the early central nervous system (Kriegstein and Alvarez-Buylla, 2009). However, these embryonic progenitors are heterogenous, and only few studies in the mouse have brough up information on their specific contribution to aNSC formation using either regional embryonic brain markers (Willaime-Morawek et al., 2006; Young et al., 2007), or signaling pathways-activated cells (Ahn and Joyner, 2005; Bowman et al., 2013; Li et al., 2013). The emerging idea is that adult neurogenic regions seem to be heterogenous regarding stem cells origin.
In both mouse and zebrafish, two subtypes of neural progenitors compose the embryonic brain at a given time point. The first progenitor population corresponds to actively neurogenic neural precursors or “proneural clusters/compartement cells”, while the second consists of NE progenitors in which neurogenesis is delayed -also called “progenitor pools/boundary cells” -(Stigloher et al., 2008). These progenitor subtypes differ in location, markers expression, sensitivity to Notch and time of recruitment, and they generate distinct neuronal populations in the developing brain. However, their relative contribution to the generation of adult neural stem cells remains unknown. The aim of this paper is to determine the respective contribution of these embryonic neural progenitor subtypes to the formation of the adult germinal zone in the zebrafish pallium.

Actively neurogenic progenitors express the *Hairy/E(spl)* gene *Hes5/her4* (Dong et al., 2012b; Takke et al., 1999). Thus, to investigate their contribution to stem cells formation, we took advantage of the *her4:ERT2CreERT2* (Boniface et al., 2009) and the *ubi:loxGFPloxmCherry* (Mosimann et al., 2011) zebrafish lines. Tamoxifen treatment on double transgenic embryos allows lineage tracing actively neurogenic progenitors expressing *her4* at any time point of life. By total recombination experiments of the *her4*-positive population at different time points during development, we observed that these only generated a subset of the adult pallial germinal zone, highlighting the heterogenous embryonic origin of aNSCs. Then, to locate the embryonic progenitor populations generating the remaining aNSCs of the pallium, we combined clonal analyses using a *ubi:CreERT2* line (Mosimann et al., 2011) and mosaic brainbow recombinations (Livet et al., 2007; Pan et al.,

2013b), with restricted spatial recombinations using caged-cyclofen coupled with laser activation (Sinha et al., 2010). These approaches revealed a restricted progenitor pool at the telencephalic dorsal midline as the complementary source of pallial aNSCs. Finally, to gain insight into the mechanisms that may be involved in the maintenance of long-lasting progenitors from embryonic stages onwards, we investigated the role of the Notch pathway by performing pharmacological treatments with a γ-secretase inhibitor applied at different time points during development. Strikingly, Notch proved necessary for the maintenance of actively neurogenic progenitors and their descendent aNSCs, but not for the maintenance of the dorsal midline progenitor pool.

Together, these experiments allowed us to conclude on several fronts: aNSCs origin (results – section 1), the progenitor maturation steps involved in their generation (results - section 2), and the organization and the development of the zebrafish pallium itself (results - section 3).

The main results of the *Dev Cell* publication regarding aNSCs origin are:

- It exists a continuum between the different neural progenitors populations and the pallial aNSC in the zebrafish pallium (Figure 29)
- The aNSCs of the dorso-medial pallium derive from early actively neurogenic progenitors that contribute to pallial formation all along development. These actively neurogenic progenitors express *her4* and are maintained by Notch signaling from embryonic stages onwards (Figure 29 -orange cells)
- On the contrary, the aNCSs of the lateral pallium derive from a NE “progenitor pool” located in the telencephalic roof plate, characterized by an apico-basal polarity, expression of *her6/her9* as well as signaling pathway ligands (Wnt, FGF, BMP), and displaying a Notch-independent maintenance.
- The NE cells of the roof plate are activated late during development (Figure 29 - purple cells).
- The neurogenic switch occurring in the lateral progenitors is concomitant with the emergence of *her4* expression and Notch-sensistive maintenance in this population.
- Some of the “progenitor pool” NE cells persist throughout life and generate *de novo* NSCs in the adult lateral pallium.
- The lateral and dorso-medial progenitor populations are functionally independent as they cannot repopulate each other following depletion of Notch-sensitive progenitors at juvenile stage.
- The progenitor maturation steps leading to aNSCs formation from NE progenitors display some similarities between the dorso-medial and the lateral pallial ventricular zone, even though the acquisition of the Notch-dependent maintenance may occur

respectively after and before the onset of *her4* expression in the progenitors. *(see results - section 2 for more details)*

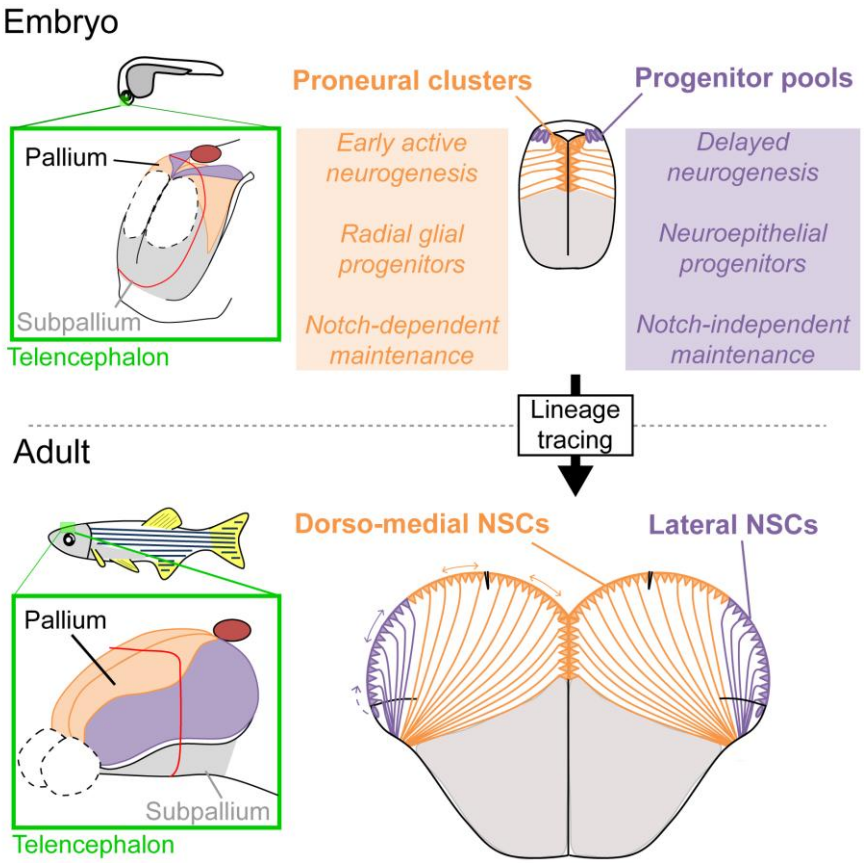


Figure 29: Graphical abstract on the embryonic origin of adult pallial neural stem cells in zebrafish

The main results of the *Dev Cell* publication regarding pallium development and regionalization are:

- Pallial neurons pile up during development with the most central pallial domain corresponding to the “oldest” region *(see results - section 3 for more details)*
- The formation of the dorso-medial and lateral pallium are heterochronous. *(see section 3 for more details)*
- The medial and dorsal pallial ventricular zones (VZ) generate the central pallium (Dc) with first the recruitement of the medial progenitors, and later, the contribution of both medial and dorsal progenitors to Dc formation. *(see results - section 3 for more details)*

- The posterior pallium (Dp) domain does not derive from the medial VZ as already proposed, but possesses the same embryonic origin than the Dl domain, together forming the lateral pallium (*see results - section 3 for more details*)
- The lateral pallium display some developmental features of the mammalian hippocampus as it develops from the progenitor population abuted to a “cortical hem-like” structure, and forms late during development.

In conclusion:

- Together, these data identify the embryonic progenitors and the processes at the origin of the aNSCs in the zebrafish pallium. They demonstrate that the two subtypes of embryonic neural progenitors (actively neurogenic proneural clusters, and progenitor pools) contribute to the generation of two spatially segregated adult pallial NSCs populations.

- Moreover, the persistence of a small NE progenitor population at the postero-lateral edge of the ventricular zone throughout life highlights differential modes of stem cells generation in the dorso-medial versus the lateral zebrafish pallium: the dorso-medial stem cell population expands by stem cell amplifying divisions, while the lateral stem cell population, in addition, is permanently fuled in by new cells. It also highlights the maturation steps present in NSCs formation.

- *her4* expression and Notch-sensitive maintenance seem to correspond to two steps important for generating adult NSCs.

- Finally, as the progenitor lineage includes the neuronal progeny, it allows us to understand how the zebrafish pallium is built during development, and the homology with pallium territories in other species.

Publication: Spatial regionalization and heterochrony in the formation of adult pallial neural stem cells

***Lara Dirian[1], Sonya Galant[1], Marion Coolen[1], Wenbiao Chen[2], Sébastien Bedu[1], Corinne Houart[3], Laure Bally-Cuif[1,*] and Isabelle Foucher[1,*]**

[1-] Institute of Neurobiology A. Fessard, Laboratory of Neurobiology and Development, CNRS UPR3294, Team Zebrafish Neurogenetics, Avenue de la Terrasse, bldg 5, F-91198 Gif-sur-Yvette, France.

[2-] Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, 2213 Garland Ave, Nashville, TN, 37232, USA.

[3-] Medical Research Council Centre for Developmental Neurobiology, King's College London, London SE1 1UL, UK.

*correspondance to bally-cuif@inaf.cnrs-gif.fr, foucher@inaf.cnrs-gif.fr

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1.2 Abstract

Little is known on the embryonic origin and related heterogeneity of adult neural stem cells (aNSCs). We use conditional genetic tracing, activated in a global or mosaic fashion by cell type-specific promoters or focal laser uncaging, coupled with gene expression analyses and Notch invalidations, to address this issue in the zebrafish adult telencephalon. We report that the germinal zone of the adult pallium originates from two distinct subtypes of embryonic progenitors and integrates two modes of aNSC formation. Dorso-medial aNSCs derive from the amplification of actively neurogenic radial glia of the embryonic telencephalon. On the contrary, the lateral aNSC population is formed by step-wise addition at the pallial edge from a discrete NE progenitor pool of the posterior telencephalic roof, activated at post-embryonic stages and persisting life-long. This dual origin of the pallial germinal zone allows the temporally organised building of pallial territories as a patchwork of juxtaposed compartments.

1.3 Introduction

Adult stem cells are multipotent at the single cell level and self-renew, sustaining the persistent generation of differentiated progeny in adult organs. The recent characterization of stem cells in the vertebrate adult brain (adult neural stem cells, aNSCs) brought new insights on neuron formation and plasticity, and adult neurogenesis is intensively studied for its role in brain homeostasis, animal behavior or brain repair (reviewed in (Imayoshi et al., 2009)). Although general features of aNSCs emerge, accumulating evidence also highlights profound heterogeneities within and between aNSC populations(DeCarolis et al., 2013; Giachino et al., 2013; Lugert et al., 2010). These may pertain to the existence of fluctuating NSC states, distinct local environments, or cell-intrinsic programs, all of which remain incompletely deciphered (Alvarez-Buylla et al., 2012).

Two main aNSC pools are constitutively active in the mouse telencephalon: the sub-ependymal zone of the lateral ventricle (SEZ) and the sub-granular zone of the hippocampus (SGZ). In both domains, recent evidence suggests that NSC “history”, i.e. the cell lineage involved in its generation and positioning, correlates to some extent with aNSC fate (De Marchis et al., 2007; Merkle et al., 2007; 2014). In this context, defining the lineages leading to aNSC formation would greatly help understand the impact of NSC formation on aNSC properties. In the mouse, a lineage relationship was proposed between embryonic neuroepithelial cells (NE cells), radial glial cells (RGCs) and aNSCs: first, overexpression of intracellular active forms of Notch in NE cells increases the generation of RGCs and postnatal periventricular and parenchymal astrocytes, some of which may be aNSCs (Dang et al., 2006; Gaiano et al., 2000); second, lineage tracing analyses of postnatal RGCs shows that their progeny contains some SEZ aNSCs (Merkle et al., 2007; 2004). Discrete lineage information was also obtained from genetic tracing based on patterning markers expression (Nkx2.1, Gsh2, Emx1, Dbx1, Gli1) in the developing mouse brain(Ahn and Joyner, 2005; Li et al., 2013; Young et al., 2007). However, little attention was paid to the early localization of the stem cell-generating progenitors within the domains traced. If the link between embryonic and adult progenitors is commonly accepted, it remains largely unknown from which specific embryonic (sub)population, how and when aNSC populations are established.

In both mouse and zebrafish, two types of neural progenitors were described in the early developing CNS (Baek, 2006; Stigloher et al., 2008). The first progenitor population corresponds to proliferative precursors actively engaged in neurogenesis. These progenitors express Notch target genes of the *E(spl)* family (*her* in zebrafish, *Hes* in mammals), such as *her4* in zebrafish or its ortholog *Hes5* in mouse, involved in neural progenitor maintenance. They are intermingled with commited neuroblasts within competent domains likely equivalent

to the “proneural clusters” of flies, where they are singled-out by Notch-dependent lateral inhibition. In mouse, these progenitors also display oscillating expression of another *E(Spl)* gene, *Hes1 (her6*, in zebrafish)(Shimojo et al., 2008). The second progenitor subtype (“boundary cells” or “progenitors pools”) consists in NE cells found at neural tube boundaries. These domains, such as the midbrain-hindbrain boundary or the Zona Limitans Intrathalamica (Geling et al., 2003; Scholpp et al., 2009) often act as signaling centers (Kiecker and Lumsden, 2005). Progenitors in these locations undergo delayed neurogenesis, and rely on a partially different set of zebrafish Her/mouse Hes transcription factors (e.g. Her5/Hes7, Her6/Hes1, Her9/Hes4) expressed at high and stable levels in a non-canonical manner independent of Notch signaling (Baek, 2006; Geling et al., 2003). The long-lasting maintenance of “proneural cluster” and “progenitor pool” neural progenitors, and their respective contribution to the generation of aNSCs, has never been directly assessed.

To address this question, we took advantage of the zebrafish adult pallium, where an extended layer of aNSCs covering the entire pallial ventricular zone (VZ) sustains widespread adult neurogenesis. aNSCs of the zebrafish pallium resemble mouse aNSCs in their identity and properties: they are RGCs, are mainly quiescent (Adolf et al., 2006; Chapouton et al., 2010a; Ganz et al., 2010; Grandel et al., 2006; Rothenaigner et al., 2011), and react to injury (Kishimoto et al., 2012; Kroehne et al., 2011; März et al., 2010; Zupanc and Sîrbulescu, 2011). Fish and mouse aNSCs also share molecular markers and express the canonical Notch target gene *her4*/*Hes5* (Basak and Taylor, 2007; Chapouton et al., 2010b; Ganz et al., 2010; Lugert et al., 2010). Using Cre-mediated conditional genetic tracing of telencephalic progenitors at successive time points during embryonic and post-embryonic development, we reveal that the two embryonic neural progenitor subtypes cooperate to build the pallial aNSC population. However, their contributions remain spatially segregated and strongly heterochronous. This unexpected bimodal process imprints a dynamic organization to the adult pallial GZ and influences building of pallial territories.

1.4 Results

1.4.1 *her4*-positive embryonic progenitors generate adult NSCs of the dorso-medial pallium

From late embryonic stages -2 days post-fertilization (dpf)- onwards, the germinal zone (GZ) of the pallium is composed of neurogenic RGCs aligned along the pallial ventricle (Figure S1A) and expressing *her4/Hes5* (Chapouton et al., 2010a; Dong et al., 2012). To appreciate the contribution of embryonic neurogenic progenitors to the pallial aNSC

population, we therefore set up a genetic strategy to follow in time and space the progeny of 2dpf *her4*$^{+}$ progenitors, using a driver line expressing a tamoxifen-inducible Cre recombinase (ERT2CreERT2) under control of a 3.4kb *her4* promoter fragment (Figure 1A). As revealed by the colocalized *her4* and *cre* expression, this line faithfully recapitulates *her4* expression in presumptive pallial progenitors at embryonic, larval and juvenile stages (Figure S1B-D and not shown). Using *her4:ERT2CreERT2* (Boniface et al., 2009) and the ubiquitous reporter line *ubi:switch* (*Tg(-3,5ubi:loxP-GFP-loxP-mCherry)*(Mosimann et al., 2010), we could permanently label with mCherry the progeny of *her4*$^{+}$ cells by the addition of 4-OHT (hydroxy-tamoxifen, activating ERT2CreERT2) at any developmental stage (Figure 1A). We did not observe any recombination without 4-OHT, arguing for reliability of this genetic system (Figure S1E).

We treated 2dpf embryos with a pulse of 4-OHT (*her4:ERT2CreERT2;ubi:switch*, T(2dpf), thereafter called *her4*$^{switch,T(2dpf)}$) and analyzed mCherry distribution within the pallial GZ and telencephalic parenchyma at 3 months post-fertilization (mpf) (Figure 1A). Unexpectedly given the large distribution of *her4*$^{+}$ RGCs in the embryonic pallium, recombined cells appeared confined to a dorso-medial subdomain of the adult pallium (Figure 1B,C). In this territory, the mCherry^{+} population included most if not all VZ cells, expressing RGC markers such as Glutamine Synthase (GS) (Figure 1D) and previously shown to possess NSC properties (Chapouton et al., 2010a; Rothenaigner et al., 2011). In the parenchyma, mCherry^{+} cells expressed the neuronal marker HuC/D (Figure 1F). These results show that dorso-medial aNSCs derive from *her4*$^{+}$ embryonic RGCs present at 2dpf, suggesting that at least some of these actively neurogenic progenitors self-renew during pallium development until adult stage to generate aNSCs. At the population level, these RGCs are bipotent as they give rise to both RGCs and neurons. Second, neither aNSCs nor neurons in the lateral pallial regions are derived from cells expressing *her4* at 2dpf (Figure 1E,F). Finally, pallial regionalization appears set and maintained from very early stages of development, as there is very little cell mixing between the mCherry^{+} and mCherry^{-} domains (Figure 1F).

1.4.2 aNSCs of the lateral pallium originate from a discrete progenitor population at 2dpf

These results prompted us to search for the embryonic progenitors generating aNSCs and neurons of the lateral pallium. To estimate the size of this early progenitor population, we first conducted sparse clonal analysis with the *ubi:creErt2* line (Mosimann et al., 2010). *ubi:creErt2;ubi:switch* embryos (*ubi*^{switch}) were 4-OHT-treated at 2dpf to generate a few

recombination events randomly distributed within the pallium (Figure 2A). At adulthood, compared to the dorso-medial domain where small clones were visible (Figures 2B,C, S2A,B, asterisks), a systematic pattern of large and complementary clones built most of the lateral VZ (and derived neurons). From 12 lateral clones observed, three subtypes could be defined. The first category (e.g. Clone 1) (n=3) corresponds to elongated clones running dorsally and abutting dorso-medial aNSCs and neurons. They reach the telencephalon-olfactory bulb junction anteriorly and terminate posteriorly at approximately two-thirds of the pallium length. The second category (e.g. Clone 2) (n=4) contains very long clones reaching from the lateral edge of the pallium anteriorly to a dorso-posterior location. The third category (e.g. Clone 3) (n=4) is made of clones restricted to the posterior lateral edge of the pallium. The restricted number of clone categories obtained, and their complementary patterns, suggest that they highlight the fate of distinct progenitors and that few progenitors at 2dpf are at the origin of the lateral pallium following a massive amplification. Quantification of the number of RGCs per clone in the dorso-medial versus lateral pallium further confirmed hugely different amplification rates: lateral clones were composed of 40 times more RGCs than clones located in the dorso-medial domain (Figure S2C), while no cell death was observed in both areas along this process (not shown).

Next, we backed up these results using the brainbow technology (Pan et al., 2013; 2011) to visualize the contribution of each embryonic pallial progenitor to the adult GZ and the relative organisation and size of clones. We used the zebrafish *hsp* promoter ubiquitously driving Cre expression upon heat-shock -and leading to efficient recombination in all pallial cells with very little leakiness (Figure S2D)- and recombined *hsp70:Cre*;*ubi:zebrabow* embryos at 2dpf (*hsp70*$^{zebrabow,\ HS(2dpf)}$) (Figure 2D). Supporting our interpretation, the resulting adult lateral pallium appeared reproducibly composed of only few large adjacent clones running all along the anterior/posterior axis of the telencephalon (Figure 2E) with no obvious cell mixing nor clonal contraction (Figure 2E,F). In contrast, clones in the dorso-medial pallium did not extend along the antero-posterior axis of the VZ, were very numerous, and strongly intermingled (Figure S2E). Similar results were observed with recombination at 1dpf (data not shown).

Altogether, these experiments reveal that the dorso-medial and lateral VZ exhibit distinct clonal behaviors: dorso-medial aNSCs derive from a large number of embryonic progenitors, each of them undergoing a small amplification; in contrast, the lateral aNSC population is formed from a small number of progenitors that later massively amplify, in an organised manner along the antero-posterior and medio-lateral axes (Figure 2G).

1.4.3 Embryonic progenitors fated to generate aNSCs of the lateral pallium are located at the telencephalic roof plate at 30hpf

We next aimed to locate the discrete embryonic progenitor population generating aNSCs of the lateral pallium. We observed in *ubi*$^{switch,T(2dpf)}$ and *hsp70*$^{zebrabowHS(2dpf)}$ animals that lateral aNSC clones always reached the lateral VZ edge (Figure S2B), suggesting that they could be growing from this location. In fitting with the everted structure of the zebrafish adult pallium (Folgueira et al., 2012), the lateral VZ edge corresponds to the attachment point to the tela choroïdea (Figure S1A). To search for *her4*$^{-}$ progenitors in an equivalent location in early embryos, we analyzed 3D reconstructions of the prosencephalon at the onset of the eversion process (1-1.5dpf, 30hpf) in *her4:GFP* transgenic fish (Yeo et al., 2007). GFP and PCNA expression revealed a *her4*$^{-}$/PCNA^{+} population dorsally just anterior to the epiphysis (Figure 3A). Within the telencephalon, this posterior roof plate was described as a region where eversion progresses and the tela choroïdea expands (Figure S3A). At 2 and 5dpf, accordingly, the *her4*$^{-}$/PCNA^{+} progenitor population was located at the lateral edge of the embryonic pallial VZ (Figure S3B).

To directly fate map the posterior telencephalic roof plate in the absence of a specific promoter, we took advantage of caged-cyclofen, a tamoxifen analog that can be activated using a 405nm laser beam (Sinha et al., 2009; 2010). We treated *ubi:creErt2;ubi:switch* embryos with caged-cyclofen and locally uncaged this compound in the posterior telencephalic roof plate in embryos at 1-1.5dpf (24-30hpf, Figure 3B). Caged-FITC-injected embryos uncaged in the same conditions and analyzed immediately after uncaging confirmed that activation was restricted to the roof plate (Figure 3C). In two cases (n=8 uncaged animals surviving to adulthood), we could obtain at 1.5mpf large mCherry^{+} lateral clones running along the antero-posterior axis, similar to the clones recovered in previous analyses (Figure 2B,C) and including the lateral VZ (Figure 3D,E). Conversely, lateral clones were never observed after uncaging in regions more anterior or more lateral (n=40 in total). Thus the posterior aspect of the embryonic telencephalic roof plate contains progenitors at the origin of lateral pallial aNSCs (and of the lateral pallium). The observed low frequency of lateral clones obtained by uncaging could be explained by the mosaïcism of the *ubi:creErt2* line, in which Cre expression diverges from embryo to embryo (L.D. unpub.), and/or by the small number of progenitors at the origin of the lateral aNSCs.

1.4.4 The embryonic source of lateral pallial aNSCs is a new “progenitor pool” subtype of signaling neuroepithelial progenitors

We next assessed the identity of the embryonic progenitors fated to lateral pallial aNSCs. Using induction time points distributed from the neural plate stage until 2dpf in

her4^{switch} fish, we first showed that these progenitors did not transiently express *her4* during embryogenesis: lateral pallial aNSCs were never mCherry^{+} following early recombinations (Figures 4A, S4A-C).

To assess whether this population harbored characteristics of the "progenitor pool" subtype, we analysed its expression of non-canonical *E(spl)* genes and signaling factors. Strong expression of *her6* and *her9*, as well as *wnt8b*, *wnt3a*, *fgf8*, and *bmp6,* was found in the appropriate location (Figure 4B-D). Further, cells in this territory did not express radial glia markers such as *blbp* and *gfap*, displayed an apico-basal polarity examplified by the ventricular distribution of ZO1 immunostaining (Figure S4D), and were positive for NSC markers such as *sox2* and *musashi1* (Figure S4E), altogether identifying them as neuroepithelial progenitors (NE).

In the early zebrafish neural plate, Notch signalling is required for progenitor maintenance within "proneural clusters" but not "progenitor pools" (Geling et al., 2004; Stigloher et al., 2008). To confirm that the embryonic progenitors fated to dorso-medial versus lateral pallial aNSCs respectively belong to these two progenitor subtypes, we took advantage of the gamma-secretase inhibitor LY411575 to conditionally inhibit Notch signal transduction (Rothenaigner et al., 2011). *her4*$^{switch,T(1dpf)}$ recombined embryos were treated with LY411575 at 2dpf, and analysed at 5dpf to directly access cell fate (Figure 4E). In control brains, PCNA^{+}/mCherry^{+} progenitors lined the ventricule and HuC/D+/mCherry^{+} neurons were present in the parenchyma (Figure 4F,F'). In the dorso-medial region of the pallium of LY-treated embryos, the PCNA^{+}/mCherry^{+} ventricular progenitors were totally missing and almost all mCherry^{+} cells were neurons (Figure 4F,F''',G). Thus, the early *her4*$^{+}$ progenitors presumably prematurely differentiated into neurons, responding to Notch inhibition as proneural clusters do. In striking contrast, laterally, the mCherry^{-} progenitor population was still present (even increased in size) following Notch blockade (Figure 4F",F'''',G). Thus, as for "progenitor pools", Notch is not involved in the maintenance of embryonic progenitors fated to lateral pallial aNSCs. Altogether, the molecular and cellular characterization of embryonic progenitors generating the dorso-medial versus lateral pallial aNSCs, and their different Notch reponsiveness, identify them respectively as "proneural cluster" versus "progenitor pool" subtypes.

1.4.5 Late growth and activation of the progenitor pool generating the lateral pallial GZ impart heterochrony to pallium development

Because classical "progenitor pools" are not immediately engaged in embryonic neurogenesis, while "proneural clusters" are (Stigloher et al., 2008), the dual origin of aNSCs may impact the developmental timing of pallial domains. To address this issue, we compared

the growth of each progenitor population and pallial territory in *her4*$^{switch,T(2dpf)}$ fish analyzed at 5dpf, 15dpf and 1.5mpf (Figure 5A). In striking contrast with the adult pallium, the mCherry$^-$ domain was largely under-represented at larval and juvenile stages: at 5dpf, the ventricular zone (VZ) was quasi-exclusively composed of proliferating progenitors expressing mCherry (Figures 5B,B',S5A). Only a small number of lateral mCherry$^-$ progenitors could be identified, all of them actively proliferating (Figure 5B", white arrow, Figure S5A,S5B). Just underneath in the parenchyma, very few mCherry$^-$ cells expressed the neuronal marker HuC/D (Figure 5B", white asterisks). At consecutive stages, the lateral progenitors amplified and were detected at 15dpf as a compact pool of PCNA$^+$/mCherry$^-$ cells at the lateral VZ edge (Figures 5C,C", S2A,B). This sharply constrasted with the significant decreased proliferation rate and limited expansion of the dorso-medial population, highlighting the differential growth of the two pools at that stage (Figure 5C,C', S5A,B). Concomitantly to progenitor amplification in the lateral pool, a large number of underlying pallial mCherry$^-$ neurons appeared in the lateral pallium (Figure 5C"). At 1.5mpf, we observed a 35% decrease in the proportion of proliferating progenitors in both the dorso-medial and lateral populations, but in the latter a strong proliferating pool was still maintained at the lateral VZ margin (Figures 5D-D", S5A,B).

These observations reveal a strong heterochrony in the amplification of the two pallial aNSC populations, and suggest a direct impact on the delayed generation of lateral pallial neurons. To verify the lineage relationship between NSCs and neurons in the lateral pallium, we analyzed at larval (4dpf) and juvenile (15dpf) stages some lateral clones generated in *ubi:creErt2;ubi:switch* fish at 2dpf (see Figure 2A-C). While lateral clones at 4dpf were largely composed of progenitors (Figure S5C,C'), they contained many neurons at 15dpf (Figure S5C,C''). Thus, lateral progenitors mainly undergo symmetric (amplifying) divisions and a very low neurogenic potential until at least 4dpf, after which stage the population both expands and generates neurons, progressively building the lateral pallium. BrdU pulse experiments conducted at 5dpf further indicated that, compared with *her4*$^+$ progenitors, the specific amplification behavior of *her4*$^-$ lateral progenitors at larval stages was not linked with accelerated cell cycle kinetics (Figure S5D,E), rather reflected distinct progenitor properties.

1.4.6 The progenitors fated to the lateral pallial aNSC population progressively switch on *her4* expression and become Notch-sensitive from juvenile stages onwards.

her4 is expressed in all RGCs of the adult zebrafish pallium, including the lateral population (Chapouton et al., 2010a). Given that lateral aNSCs derive from *her4*$^-$ embryonic NE cells, this raises the question of the onset of *her4* expression in this population, and of the potential persistence over time of a lateral aNSCs-generating progenitor pool. To address

these points, we recombined *her4*^{switch} fish with 4-OHT at different time points of larval and juvenile development and compared the number of lateral mCherry^{+} RGCs in each condition, from a fixed morphological landmark (the medial pallial sulcus -sulcus ypsiloniformis-) located within the dorso-medial domain (Figure 6A,B, yellow arrowheads). Further to our initial observations (Figure 4A), the mCherry expression boundary appeared remarkably stable in adult brains resulting from 4-OHT treatment up to 3.5dpf, demonstrating that progenitors fated to lateral aNSCs remain *her4*$^{-}$ at least until 3.5dpf (Figure 6A, left panel). However, this boundary shifted laterally with 4-OHT recombinations conducted at 5 and then 15dpf (Figure 6A, middle and righ panels). Quantifications of the number of mCherry^{+} RGCs (assessed using GS expression) confirmed these observations (Figure 6B). Thus, lateral pallial progenitors progressively express *her4* de novo from 5dpf onward. To test whether this was correlated with Notch-dependency, we performed a LY411575 treatment on 15dpf wildtype fish (Figure 6C). In contrast with the effect of Notch blockade at 2dpf (Figure 4F,G), late LY411575 applications resulted in the depletion of most dorso-medial and lateral pallial progenitors (Figure 6C).

Altogether, these results highlight the progressive and coincident emergence of neurogenic potential, *her4* expression and Notch-dependent maintenance in lateral progenitors from late larval stages onwards.

1.4.7 A minute population of NE progenitors persists throughout life at the postero-lateral edge of the pallial VZ and ensures the continuous generation of neurogenic aNSCs

Close inspection of juveniles treated with LY411575 from 15dpf onwards revealed that a small pool of proliferating progenitors was refractory to treatment and consistently maintained posteriorly at the lateral telencephalic VZ edge (Figure S6). This pool appeared negative for glial markers and for mCherry (in experiments conducted with *her4*$^{switch,T(2dpf)}$ recombined fish) (Figure S6), indicating that it originates from *her4*$^{-}$ progenitors and maintains NE characteristics. As a first approach to test whether this pool could serve as an aNSC source, we assessed its long-term fate following the depletion of Notch-sensitive progenitors. We treated *her4*$^{switch,T(2dpf)}$ juveniles at 15dpf for 4 days with LY411575 and analysed their telencephalon at adult stage after a 3-month chase (Figure 6D). Compared to control fish, most of the GS^{+} RGCs were absent in the dorso-medial pallium (mCherry^{+}), where neurons directly abutted the ventricle (Figure 6D',D'''). In contrast, the lateral VZ was partially repopulated by RGCs and displayed an organization similar to control fish (Figure 6D'',6D''''). Interestingly, the mCherry^{-} RGC population was restricted to the lateral pallial domain and did not extend over the mCherry^{+} dorso-medial area. An 8-month chase gave

the same result (not shown). Thus, the Notch-independent NE pool at juvenile stage can behave as aNSCs source to build lateral pallial domains, but cannot reconstitute the dorso-medial pallial aNSC population.

To determine whether a small lateral NE progenitor pool was maintained at the lateral edge of the pallial VZ until adult stage, we analysed cellular organisation and the expression of progenitor pool markers in this location. At posterior levels, some $GS^{-}/PCNA^{+}$ cells were visible laterally, at the junction between the lateral pallial VZ and the tela-choroïda. These cells are negative for *her4* but some express *her9* and *wnt3a* (Figures 7A,7B and S7A). The neurogenic marker *ascl1b*, orthologous to mammalian *Mash1*, is also expressed in this region, indicative of ongoing neurogenesis (Figure S7B). These patterns were consistent at all stages examined (Figure S7C-E). To determine whether the pool of NE cells persisting at late stages participated in the formation of the adult pallium, we recombined $her4^{switch}$ fish at 1.5mpf and analysed mCherry expression after a long chase of 3.5 months. No obvious $mCherry^{-}$ domain was generated at anterior and medial telencephalic levels during this time (not shown). However, at posterior levels, an obvious $mCherry^{-}$ domain was apparent that included both proliferating progenitors and neurons (Figure S7F). Together, these results highlight that some progenitor NE cells are maintained throughout life at the lateral pallial edge, where they act as long-lasting generators of lateral pallial aNSCs and neurons.

1.5 Discussion

The genetic fate-mapping studies conducted here bring unprecedented information on aNSCs formation. To our knowledge, the existence of a continuum between embryonic neural progenitors and aNSCs is here established for the first time over the entire extent of the pallial GZ, complementing and extending the tracing experiments performed in mouse with regional identity markers (Ahn and Joyner, 2005; Li et al., 2013; Young et al., 2007). Further, and most importantly, our results reveal that pallial aNSCs are heterogenous in terms of their embryonic origin and result from the recruitment of two contrasting progenitor populations, which differ in their location, gene expression, properties and growth mode. Because these progenitors do not mix, this dual mode of GZ formation imposes pronounced heterochrony and compartmentalization to pallium construction, which, as discussed below, is in correlation with the generation of functionally distinct neuroanatomical domains.

A major finding of our work regarding GZ formation is the dual origin of aNSCs in the zebrafish pallium. The adult pallial GZ appears composed of two distinct populations of aNSCs, which originate from different sources of embryonic progenitors that are set-up, positioned and amplified according to strikingly different developmental processes (Figure 7C). Dorso-medial aNSCs are generated by amplification from a large, finite population of *her4*$^{+}$, neurogenic pallial progenitors located along the posterior ventricular wall of the embryonic telencephalon at 2dpf, and which, at least for some of them, maintain *her4* expression until adulthood. On the contrary, lateral aNSCs arise from juvenile stages onwards by the stepwise addition at the lateral edge of the pallial VZ of RGCs turning on *her4* expression *de novo*. The latter process is initiated from a very restricted set of embryonic *her4*$^{-}$ NE progenitor and follows an antero-posterior and medio-lateral progression. This population is long-lasting, persisting in the adult at the posterior edge of the telencephalic VZ. This mechanism appears remarkably reminiscent of body axis elongation in vertebrates or long germ-band insects (Rosenberg et al., 2009), or of polarised growing organs bearing at one pole a zone of actively dividing progenitors, followed by a zone of differenciated cells. Such a “permanent conveyor belt” system is used for exemple in the fish retina and optic tectum but also in mammalian intestinal crypts (Devès and Bourrat, 2012). The clear temporal shift in the initiation of these two distinct aNSC generation processes, and their constant spatial segregation, suggest that strong converging forces ensure their cooperation to generate a seemingly uniform pallial GZ. How these two pools of embryonic progenitors are specified in the early embryo, and which mechanism triggers the late activation of the *her4*$^{-}$ NE pool, remain important issues to adress.

In parallel, our analyses of gene expression and Notch sensitivity in pallial progenitors further highlight that this dual mechanism correlates with the recruitment of different embryonic progenitor subtypes: the progenitors generating dorso-medial aNSCs display early neurogenesis, *her4* expression, and are maintained by Notch signaling; in contrast, the progenitors fated to the lateral aNSC population delay their activation and neurogenesis until post-embryonic stages, express the two non-canonical *E(spl)* genes *her6* and *her9* (Bae, 2006; Scholpp et al., 2009) but not *her4*, express signaling factors, and do not depend on Notch for their maintenance. Thus, unlike previous asumptions (Chapouton et al., 2006; Stigloher et al., 2008), aNSC generation even in a single brain subdivision such as the pallial GZ is not the prerogative of a defined embryonic progenitor subtype (Stigloher et al., 2008). Embryonic proneural clusters also derive from *her4*$^{-}$ progenitors before the neural plate stage (Kageyama et al., 2008). Likewise, at late stages, these two progenitor subtypes appear lineage-related, since some lateral progenitors progressively turn on *her4*, coinciding with their generation of the first lateral neurons, and become Notch-sensitive. In the embryonic neural tube however, the two progenitor subtypes studied here are circumscribed to distinct territories, and appear not able to repopulate each other. Whether the association of each progenitor subtype with a specific growth mode, as demonstrated here, is a general phenomenon that holds in other neural tube areas remains to be assessed.

Our study also indirectly provides novel information on pallium construction. First, our data reveal striking molecular and maturation similarities between the formation of the zebrafish lateral pallium and the mammalian hippocampus –and their respective aNSC progenitors-. A latero-medial gradient of neurogenesis drives the late formation of the hippocampus in the mouse telencephalon (Machon et al., 2007). We show that the same neurogenic gradient exists in the teleost fish but, due to telencephalic eversion, is inverted (medio-lateral) compared to mouse. The zebrafish lateral domain and the mouse hippocampus also share comparable embryonic origins: we show that the lateral pallium derives from progenitors located immediately adjacent to the dorsal telencephalic midline, a situation very similar to the mouse hippocampus, specified at the boundary of the cortical hem and futur cortex (Mangale et al., 2008; Subramanian et al., 2009). In addition, expression of *wnt3a* and *wnt8b,* marking the developing mouse cortical hem (Mangale et al., 2008; Ragsdale et al., 1998; Rash and Grove, 2011), highlights the location of lateral pallial progenitors at the roof plate of the zebrafish telencephalon. Finally, BMP signalling drives *Hes1/her6* expression and controls cell fate choices at the mouse dorsal telencephalic midline (Imayoshi et al., 2008), and both *bmp* and *her6* are co-expressed in the posterior telencephalic roof plate of the zebrafish embryo. These observations add strong ontogenetic support to the view that the lateral domain of the zebrafish pallium hosts the homologous

region of the hippocampus (Northcutt, 2005; Wullimann, 2009). In addition, the lateral pallial domain is involved in spatial and temporal aspects of learning processes in different fish (Braford, 2009; Broglio et al., 2010; Vargas et al., 2006). It is interesting to note that its delayed formation, demonstrated here, matches behavioral observations that learning in zebrafish (as in mammals) starts reliably around 3 weeks and reaches adult performance levels at 6 weeks post-fertilization (Valente et al., 2012). Finally, this study highlights the life-long persistence at the pallial VZ edge of embryonic NE progenitors able to generate new lateral RGCs. Whether such a population is also maintained in the mouse hippocampus will be interesting to assess. Thus, the specific origin and growth mode of the lateral pallium likely bears critical physiological consequences. More generally, the genetic strategy described here will provide a unique tool to overlap developmental grounds to functional pallial domains and the pallial subdivisions initially proposed based on histology.

1.6 Material and Methods

1.6.1 Zebrafish lines and staging of juvenile animals

Wild-type (AB and EKK strains) and Tg(*her4:eGFP*) (Yeo et al., 2007), Tg*(ubi:switch)* and *Tg(ubi:CreErt2)* (Mosimann et al., 2010), Tg(*her4:ERT2CreERT2*) (*Boniface and al., 2009*), *hsp:*Cre and *ubi:zebrabow* (Pan et al., 2011) transgenic zebrafish were used. Embryos/larvae up to 5dpf were maintained and staged as described (Kimmel et al., 1995). Based on size and morphological criteria (Parichy et al., 2009) and http://zfin.org/zf_info/zfbook/stages/index.html), we determined that larvea and juveniles at 5dpf and 15dpf correspond respectively to 3.8mm- and 6.3mm-long fish. Adult zebrafish were maintained using standard fish-keeping protocols and in accordance with our Institute's Guidelines for Animal Welfare.

1.6.2 4-OHT treatments and BrdU incorporation

4-Hydroxytamoxifen (4-OHT, T176, Sigma) treatment was performed as previously described (Mosimann et al., 2010) see "supplementary experimental procedures" for optimal recombination conditions. Fish were then washed 4 times, transferred into fresh embryo medium and grown as usual. *ubi*^{switch} embryos at 2dpf were placed into 5µM 4-OHT for 24h. BrdU labeling at 5 dpf was performed as previously described (Coolen et al., 2012)

1.6.3 Caged-cyclofen and caged FITC uncaging experiments

Caged-cyclofen (provided by L. Jullien, (Sinha et al., 2010)) was applied at 3.3µM for 3 hours on dechorionated 1dpf embryos. Caged-FITC (synthesized following Chen Lab protocol using CMNB-caged fluorescein SE -Life technology-) was injected at the one-cell stage. Embryos were then washed 4 times, transferred into 0.02% tricaine in embryo medium, and uncaged one by one from the dorsal side using the 405nm laser beam for 1min (upright confocal microscope, Zeiss LSM710). Washed embryos were grown in individual tanks.

1.6.4 LY411575 treatments

For LY411575 treatments, embryos and juveniles were placed in embryo medium containing 50 µM LY as indicated (MACS milteny), except for Figure 6d, where treatment was for 2 days at 50µM and 2 days at 10µM. Control embryos were incubated in embryo medium containing 0.04% DMSO. At least 4 brains were analyzed, obtained from at least two independent experiments.

1.6.5 Immunohistochemistry and *In Situ* Hybridization

Immunohistochemistry and in situ hybridization were performed as described previously (Bosco et al., 2013; Chapouton et al., 2010a; Ninkovic et al., 2005) –see Supplementary information for detailed antibodies and probes used in this study.

1.6.6 Image acquisition and 3D reconstructions

Images taken using a confocal microscope (Zeiss LSM700) were processed with the ZEN 2009 software (Carl Zeiss MicroImaging) and Photoshop CS6. Scale bars for adult brain images: 50µm, for stages up to 15dpf: 20µm. Dorsal whole-mount views of the telencephalon were taken using a Nikon macrozoom. Zebrabow brains were prepared as described in (Weissman et al., 2011) and imaged on an upright confocal (Zeiss LSM710).
Schematic 3D reconstructions of a 2dpf telencephalon, comparing the expression pattern of 4 genes, were obtained using the FreeD software (Andrey and Maurin, 2005) extrapolating results from several double ISH.

1.6.7 Cell counting

For countings the number of RGCs per clone in Figure S2C, RGCs of the clones (GS-positive/mCherry-positive cells) in the dorso-medial VZ were counted manually from 2 different telencephali countaining both medial and lateral clones. Countings were done on the entire Z-confocal stack (50µm thickness) and on the appropriate number of sections to encompass the entire clone (approximately 2-3 sections). For the lateral clones, the number of RGCs was counted manually on 3 different sections crossing the clone (one anterior, one medial, one posterior) on the entire Z-confocal stack, averaged and extrapolated depending on the number of sections where the clone was present (approximately 10-12 sections).
For cell countings in Figure 4G, 4 cryostat sections were prepared from 4 telencephali from her4switchT(1dpf) fish at 5dpf in LY411575 treated and control conditions. Proliferating cells, representing 98% of the progenitor population at 5dpf, were counted manually from the entire pallial VZ of at least 4 different sections per telencephalon.
For cell countings in Figure S5A, and B, cryostat sections were prepared from a minimum of 3 telencephali from her4switchT(2dpf) fish at each stage. PCNA-positive cellss, i.e. amplifying progenitors, were counted manually from the entire pallial VZ of at least 3 sections per telencephalon.
For the countings of BrdU cells in Figure S5E, cryostat sections were prepared from 4 telencephali from her4:GFP fish at 5dpf incubated with BrdU solution for a short pulse. The labelling index (BrdU-positive cells whithin progenitorsPCNA-positive cells) was estimated in

the her4-positive (BrdU-positive/PCNA-positive/her4-positive cells) and her4-negative (BrdU-positive/PCNA-positive/her4-negative cells) populations. Cells were counted manually from the entire pallial VZ of at least 4 different sections per telencephalon.
For cell countings in Figures 6A-B, 3 vibratome sections were analysed from a minimum of 3 telencephali from recombined her4switch fish in each condition. Ventricular glial cells from the ypsiloniformis sulcus (medial pallial sulcus) to the lateral VZ edge of the ventricular zone were counted manually from medial sections (sections showing the medial pallial dorsal and lateral sulcus).
All these countings were done on single optical plane images taken with the 40x oil objective.

1.6.8 Statistics

All experimental data were analyzed using Excel and in vivo stat (Clark et al., 2012) software **and** are expressed as mean ± 95% confidence interval (95%CI). They were all compared using one-way analysis of the variance (ANOVA, in vivo stat), followed by a post-hoc test (Bonferroni correction) only for Figures 4G and 6B. For Figure S5B, an ArcSinus transformation was performed before comparing data with a one-way ANOVA, and a post-hoc test (Bonferroni correction). Significance was set at $P < 0.05$.

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1.7 Figures

Figure 1 : *her4*-expressing progenitors at 2dpf generate adult NSCs of the dorso-medial pallium.

(A) Genetic strategy used for the time-controled fate mapping of *her4*-expressing progenitors: 4-hydroxy-tamoxifen (4-OHT) triggers ERT2CreERT2 activation in *her4*+ progenitors allowing *GFP* excision and permanent *mcherry* expression (upper panel). Experimental design to map the adult fate of early *her4*+ progenitors: 4-OHT is applied at 2dpf (*her4*$^{switch,T(2dpf)}$) and recombined animals are analyzed at 3mpf (lower panel).

(B) Dorsal view (whole-mount, anterior left) of a *her4*$^{switch T(2dpf)}$ adult telencephalon showing regionalized mCherry expression. Doted lines delineate the telencephalon (Tel) and the olfactory bulb (OB).

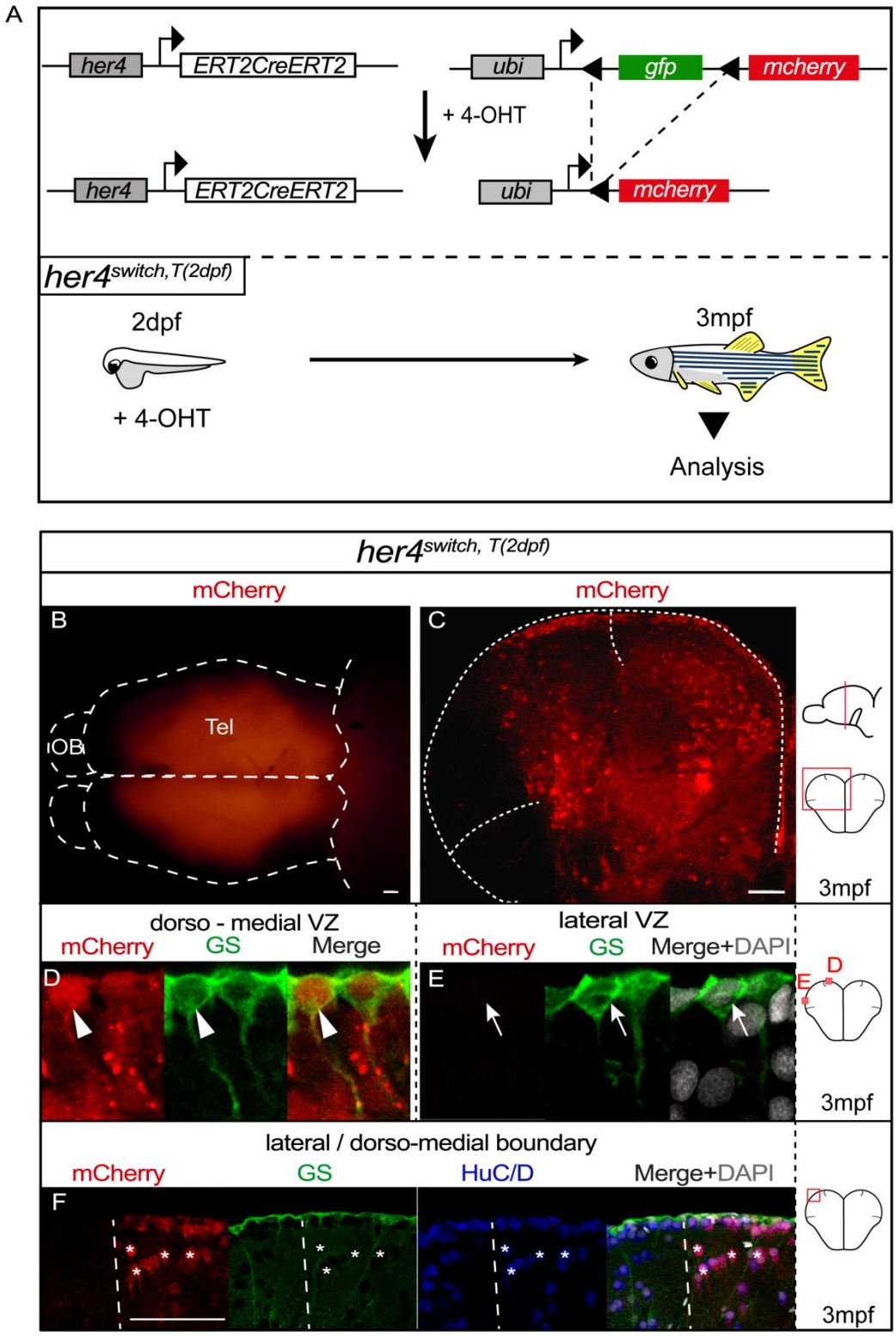
(C) Cross-section of the telencephalon in a *her4*$^{switch,T(2dpf)}$ adult, focusing on the pallium and stained as indicated. Dotted lines delineate pallial boundaries with the medial and lateral pallial sulci; one hemisphere is shown.

(D, E) High magnification of the dorso-medial (D) or lateral (E) NSCs. mCherry is expressed only in dorso-mediel RGCs expressing GS (D, arrowheads) and not in lateral RGCs (E, arrows).

(F) Magnification of the boundary (dotted line) between the dorso-medial and lateral pallial domains, showing the segregation of mCherry^{+} and mCHerry^{-} NSCs and neurons. asterisks to some mCherry+ neurons.

See also FigureS1.

Figure 1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mCherry
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Tel
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C
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dorso - medial VZ
mCherry
GS
Merge
lateral VZ
mCherry
GS
Merge+DAPI
D
E
D
E
3mpf
lateral / dorso-medial boundary
mCherry
GS
HuC/D
Merge+DAPI
F
3mpf

Figure 2: A restricted number of progenitors at 2dpf generates the lateral aNSCs following massive post-embryonic amplification.

(A) Experimental design to analyze the morphology of aNSCs polyclones generated from embryonic progenitors in the lateral pallium.

(B) Dorsal (upper panels) and lateral views (lower panels) of *ubi*$^{switch,T(2dpf)}$ telencephali (whole mount of one hemisphere, anterior left). Representative lateral clone types 1, 2 and 3 are shown (dotted lines). Asterisks highlight medial pallial clones.

(C) Cross-sections of the telencephali shown in B (section plane : yellow) and stained as indicated.

(D) Experimental design to map the adult fate of individual early pallial progenitors in single brains**.**

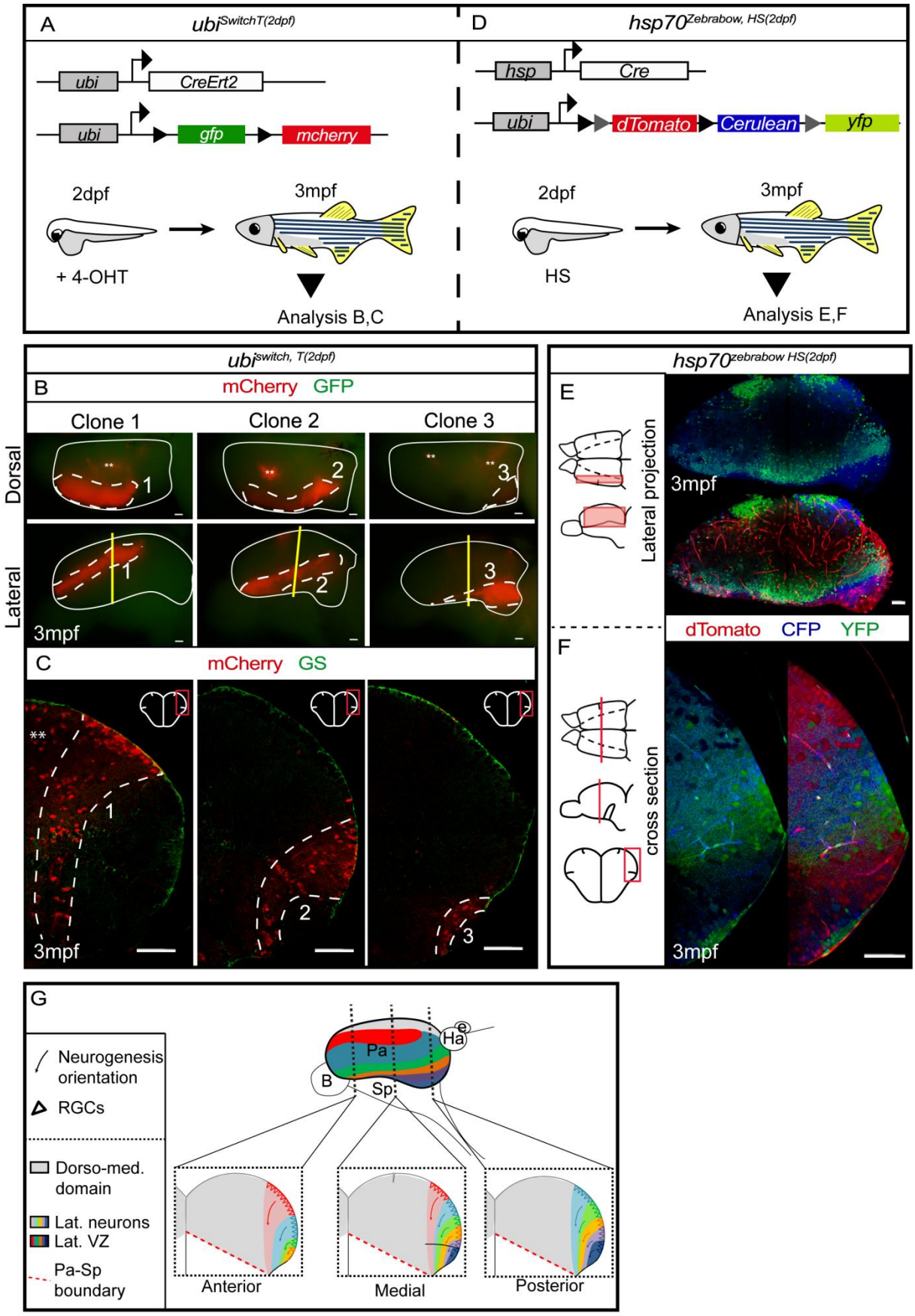
(E) Lateral projections of the adult pallium (whole-mount) in *hsp70*$^{zebrabow,HS(2dpf)}$ fish showing expression of CFP/YFP (upper panel) or CFP/YFP/dTomato (lower panel).

(F) Cross-section of the telencephalon of *hsp70*$^{zebrabow,HS(2dpf)}$ adults showing expression of CFP/YFP (left panel) or CFP/YFP/dTomato (right panel).

(G) Scheme depicting the typical morphology of lateral pallial clones after a recombination as in (A,D), and cross-sections at anterior, medial and posterior levels. Triangles to the RGCs, colored domains to the neurons generated from these progenitors, and arrows to the progression of neurogenesis.

See also FigureS2.

Figure 2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hsp
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ubi
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yfp
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B
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Clone 2
Clone 3
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E
Lateral projection
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dTomato CFP YFP
F
cross section
3mpf
G
Neurogenesis orientation
RGCs
Dorso-med. domain
Lat. neurons
Lat. VZ
Pa-Sp boundary
Pa
Ha
B
Sp
Anterior
Medial
Posterior

Figure 3: Lateral aNSCs-fated embryonic progenitors are located in the posterior telencephalic roof plate.

(A) 3D view of the prosencephalon at 1.5dpf in a *her4:GFP* embryo immunostained as indicated. Dashed line: position of the epiphysis (e), and **: posterior part of the telencephalic roof plate. Te: telencephalon.

(B) Experimental design to fate map embryonic progenitors located at the posterior telencephalic roof plate**:** caged-cyclofen ([cyclofen]) was locally photoactivated in *ubi:creErt2;ubi:switch* embryos at 1-1.5dpf using a 405nm laser beam (blue box to the laser-activated area). Recombined animals (*ubi*$^{switch,uncag(1\text{-}1.5dpf)}$) were analysed at 1.5mpf.

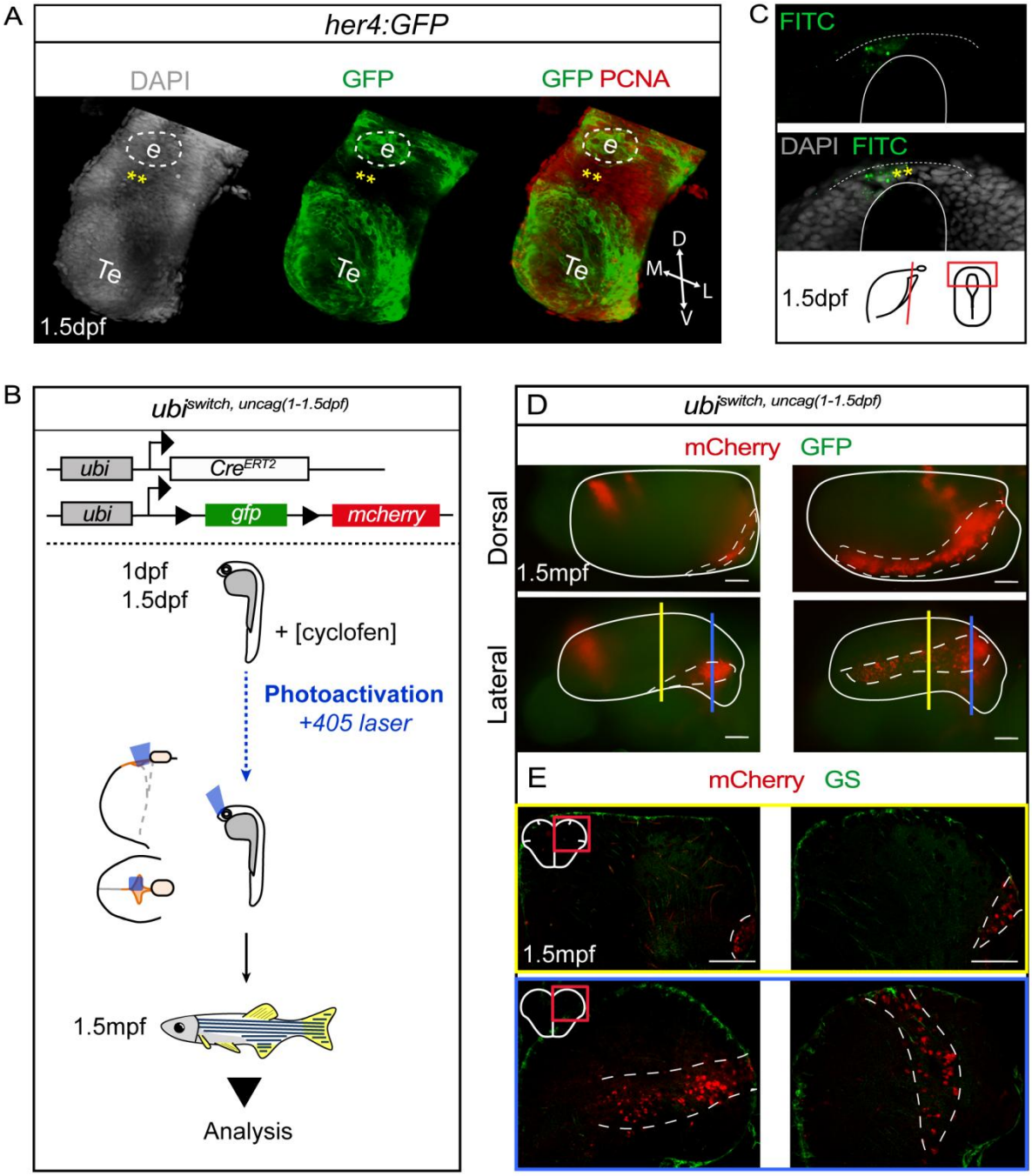
(C) Cross-section focusing on the posterior telencephalic roof plate of an embryo injected with caged-FITC and analysed immediately after uncaging. The uncaged area (asterisks) is limited to the roof plate.

(D) Dorsal (upper panel) and lateral (lower panel) whole-mount views of *ubi*$^{switch,uncag(1\text{-}1.5dpf)}$ telencephali. Dotted lines surround the pallium.

(E) Cross-sections at medial level (upper panels – yellow section plane in D) and at posterior level (lower panel – blue section plane in D) of *ubi*$^{switch,uncag(1\text{-}1.5dpf)}$ lateral telencephali stained as indicated.

See also FigureS3.

Figure 3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e
Te
1.5dpf
D
M
L
V
C
FITC
DAPI FITC
1.5dpf
B
ubiswitch, uncag(1-1.5dpf)
ubi
CreERT2
ubi
gfp
mcherry
1dpf
1.5dpf
+ [cyclofen]
Photoactivation
+405 laser
1.5mpf
Analysis
D
ubiswitch, uncag(1-1.5dpf)
mCherry
GFP
Dorsal
Lateral
1.5mpf
E
mCherry
GS
1.5mpf

Figure 4: The *her4*-negative cells at the origin of lateral pallial aNSCs belong to the "progenitor pool" subclass of embryonic neural progenitors

(A) Number of mCherry^{+} RGCs per adult telencephalic section, from the sulcus ypsiloniformis up to the edge of the VZ, after recombination at 1-10 somites, 1dpf or 2dpf of *her4*^{switch} embryos.

(B-D) Compaired expression of *her4*, *her9, her6*, *wnt8b*, *wnt3a*, *fgf8* and *bmp6* along the posterior telencephalic roof plate at 1.5dpf, revealed by fluorescent ISH without/with DAPI. Frontal (B,C) or horizontal (D) single confocal planes are shown. Dashed line : roof plate of the neural tube (or epiphysis) and plain lines : ventricle. **: roof plate. e: epiphysis, Te: telencephalon.

(E) Experimental design to assess Notch sensitivity of pallial progenitors at 2 dpf.

(F) Medial cross-sections of the telencephalon in *her4*$^{switch\text{T}(1dpf)}$ larvae treated with DMSO or LY411575. Magnification of the dorso-medial VZ (F', F"') and lateral VZ (F'', F""'), immunostained as indicated. Arrows and arrowheads highlight respectively the dorso-medial progenitors (mCherry^{+}/PCNA^{+} cells) and the lateral progenitors (mCherry $^{-}$/PCNA^{+} cells).

(G) Compaired number of pallial dorso-medial progenitors (dark gray) and of lateral progenitors (light gray) in control (DMSO) and treated (LY411575) conditions. Values are presented as mean ± 95%CI (ANOVA).

See also FigureS4.

Figure 4

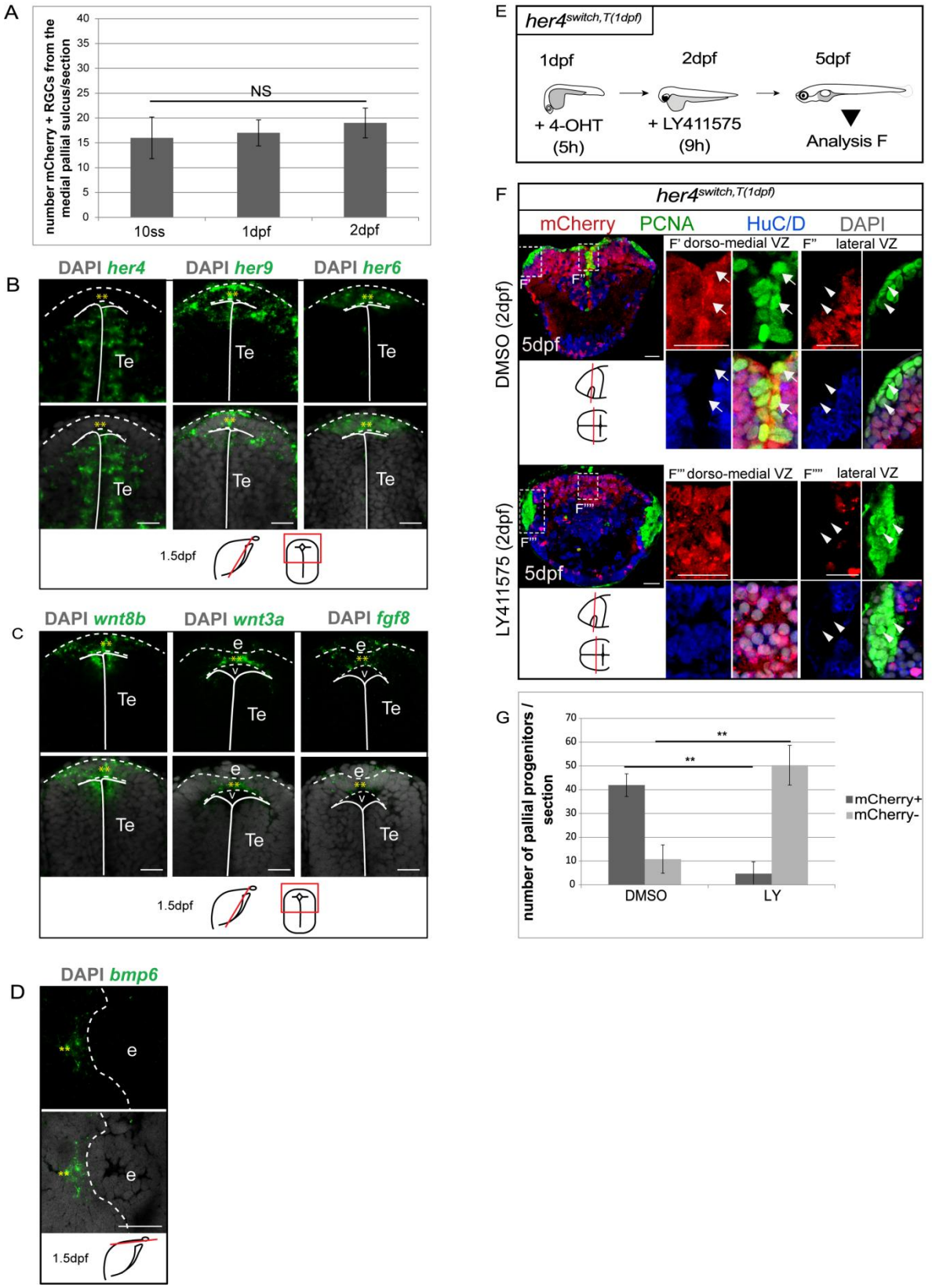

A
number mCherry + RGCs from the medial pallial sulcus/section
NS
10ss
1dpf
2dpf
B
DAPI her4
DAPI her9
DAPI her6
Te
1.5dpf
C
DAPI wnt8b
DAPI wnt3a
DAPI fgf8
e
v
D
DAPI bmp6
E
her4switch,T(1dpf)
1dpf
2dpf
5dpf
+ 4-OHT (5h)
+ LY411575 (9h)
Analysis F
F
her4switch,T(1dpf)
mCherry
PCNA
HuC/D
DAPI
F' dorso-medial VZ
F'' lateral VZ
DMSO (2dpf)
F''' dorso-medial VZ
F'''' lateral VZ
LY411575 (2dpf)
5dpf
G
number of pallial progenitors / section
mCherry+
mCherry-
DMSO
LY

Figure 5: Pallium development occurs in two heterochronic waves.

(A) Experimental design. (B-D) Cross-sections of the telencephalon in *her4*$^{switch,T(2dpf)}$ animals at 5dpf (B), 15dpf (C), and 1.5mpf (D) stained as indicated. Arrows and asterisks highlight respectively the PCNA^{+}/mCherry $^{-}$ progenitors and the first neurons of the lateral pallium. Arrowheads indicate the lateral pallial sulcus.

See also FigureS5.

Figure 5

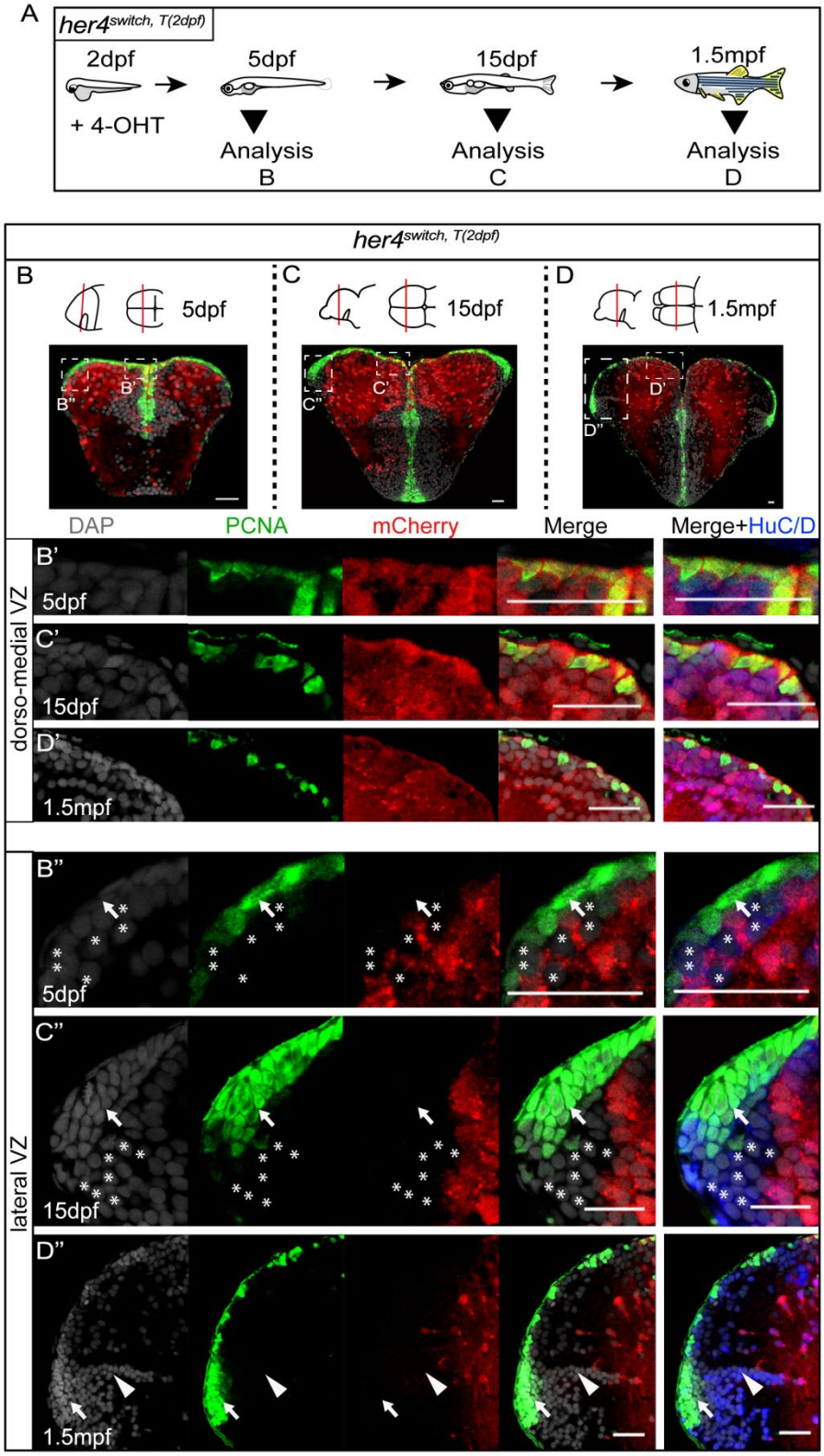

A
her4switch, T(2dpf)
2dpf
+ 4-OHT
5dpf
Analysis
B
15dpf
Analysis
C
1.5mpf
Analysis
D
her4switch, T(2dpf)
B
5dpf
C
15dpf
D
1.5mpf
DAP
PCNA
mCherry
Merge
Merge+HuC/D
dorso-medial VZ
B'
5dpf
C'
15dpf
D'
1.5mpf
lateral VZ
B''
5dpf
C''
15dpf
D''
1.5mpf

Figure 6: lateral progenitors progressively express *her4* and become Notch-sensitive at juvenile stages but maintain a cryptic boundary with the dorso-medial VZ.

(A) Adult fate of progenitors expressing *her4* at 3.5dpf (*her4*$^{switchT(3.5dpf)}$), 5dpf (*her4*$^{switchT(5dpf)}$) or 15dpf (*her4*$^{switch,T(15dpf)}$): experimental design and respective cross-sections of adult telencephali immunostained as indicated. Yellow stars and dotted lines indicate the mCherry^{+} boundary observed after recombination at different stages (single star: recombination at 2dpf, double stars: recombination at 5dpf, triple stars: recombination at 15dpf). Yellow arrowhead to the sulcus ypsiloniformis.

(B) Numbers of mCherry^{+} RGCs after recombination at 2dpf, 3.5dpf, 5dpf and 15dpf in *her4*^{switch} fish - counted from the sulcus ypsiloniformis up to the lateral edge of the VZ-. mCherry^{+}/GS^{+} RGCs make 33% of the VZ at 3.5dpf, and 85% after a recombination at 15dpf. Values are presented as mean ± 95%CI (ANOVA, *P<0.05).

(C) Notch sensitivity of pallial progenitors at juvenile stages. Experimental design and cross-sections of the telencephalon in control (upper panels) or LY411575-treated fish (lower panels) immunostained as indicated. Magnification of the dorso-medial VZ (C', C''') and lateral VZ (C'', C''''), immunostained as indicated. Arrows point to medial progenitors, arrowheads to lateral progenitors, empty arrows/arrowheads when these progenitors are depleted.

(D) Adult analysis of the telencephalon of fish treated with a Notch inhibitor at juvenile stage. Experimental design and cross-sections of the telencephalon in control (upper panels) or LY411575-treated fish (lower panels) immunostained as indicated. Arrows point to medial (mCherry^{+}) progenitors, arrowheads to lateral (mCherry^{-}) progenitors, empty arrows/arrowheads when these progenitors are depleted.

See also FigureS6.

Figure 6

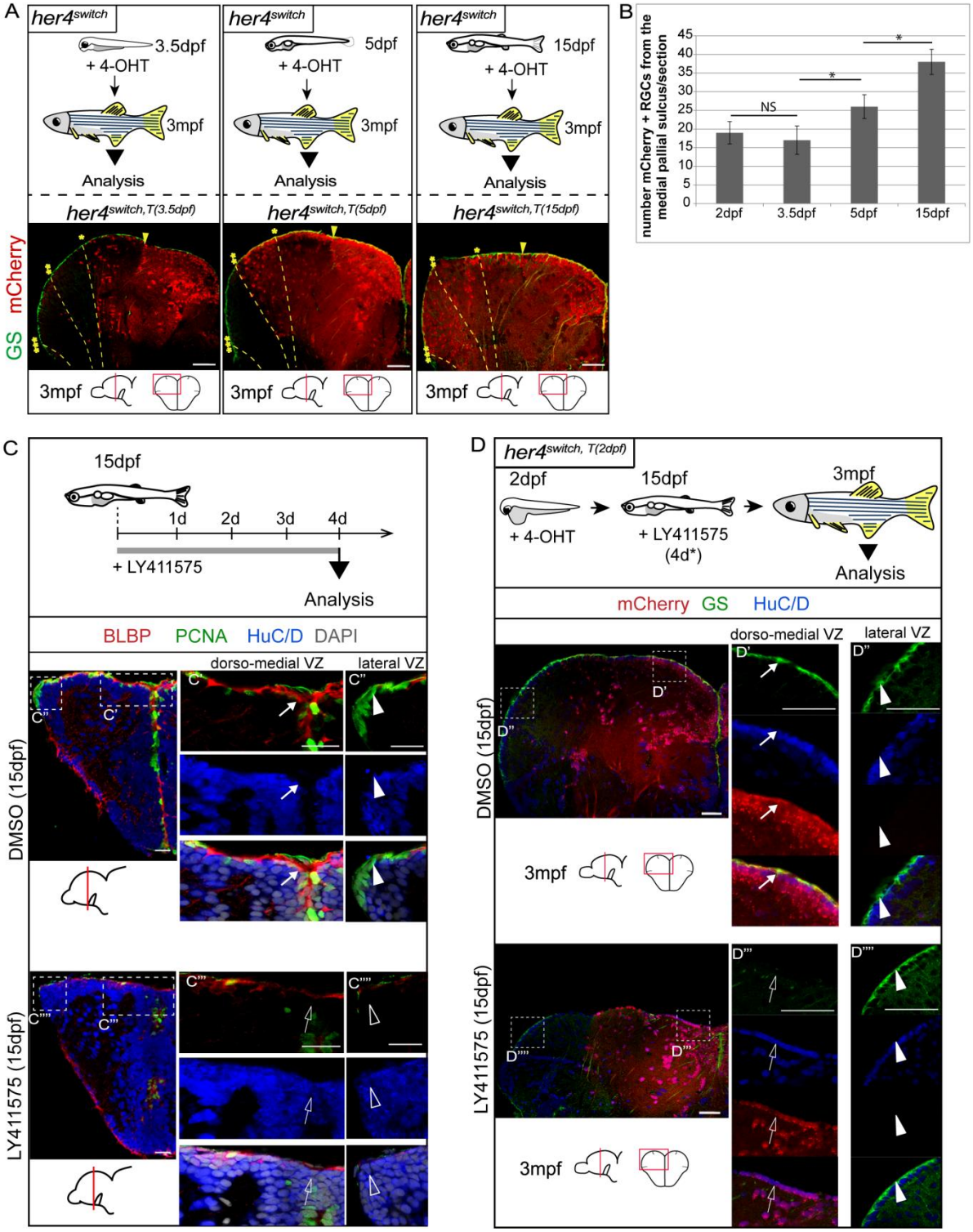

A
her4switch
3.5dpf
+ 4-OHT
3mpf
Analysis
her4switch
5dpf
+ 4-OHT
3mpf
Analysis
her4switch
15dpf
+ 4-OHT
3mpf
Analysis
her4switch,T(3.5dpf)
her4switch,T(5dpf)
her4switch,T(15dpf)
mCherry
GS
3mpf
B
number mCherry + RGCs from the medial pallial sulcus/section
NS
*
*
2dpf
3.5dpf
5dpf
15dpf
C
15dpf
1d
2d
3d
4d
+ LY411575
Analysis
BLBP PCNA HuC/D DAPI
dorso-medial VZ
lateral VZ
DMSO (15dpf)
LY411575 (15dpf)
D
her4switch, T(2dpf)
2dpf
+ 4-OHT
15dpf
+ LY411575
(4d*)
3mpf
Analysis
mCherry GS HuC/D
dorso-medial VZ
lateral VZ
DMSO (15dpf)
LY411575 (15dpf)
3mpf

Figure 7: Dual embryonic origin of pallial aNSCs and persistence of adult NE cells.

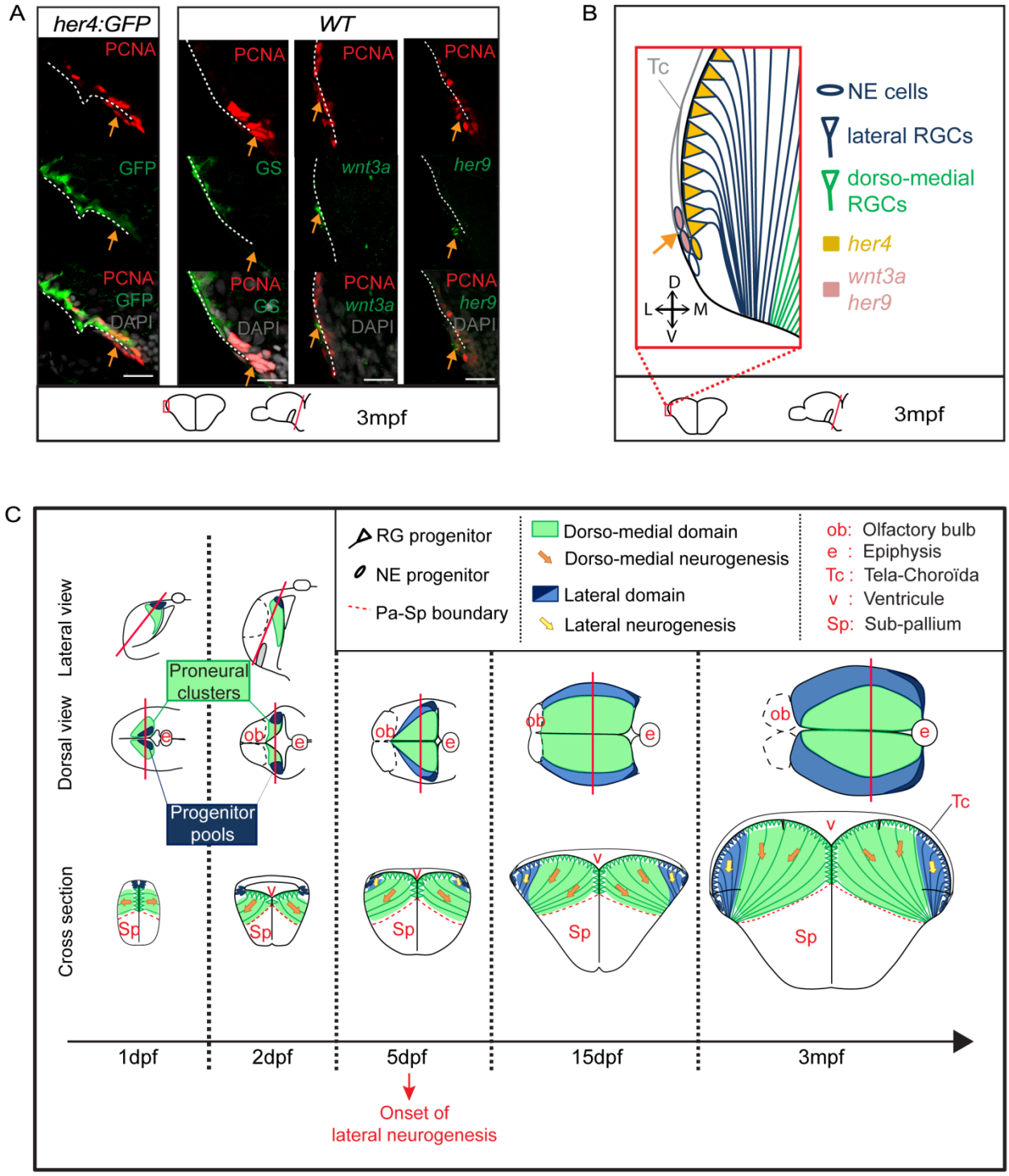
(A) Posterior cross sections showing the lateral edge of the adult VZ in *her4:GFP* fish (left panel) or *WT* fish (right panels) stained as indicated. Arrows to the *her4*$^{-}$/PCNA^{+} NE pool of progenitors.

(B) Scheme of the lateral edge of the adult posterior pallial VZ (as shown in (A)) depicting RGCs (blue triangles), NE cells (blue), *her4* expression (yellow), and *wnt3a*/*her9* expression (pink). The *her4*$^{-}$ NE pool contains *wnt3a*$^{+}$ and/or *her9*$^{+}$ proliferating progenitors at the junction between the tela-choroïdea and the posterior pallial VZ edge.

(C) Summary of pallium formation: The dorso-medial pallial NSCs and neurons (green) derive from embryonic neurogenic progenitors. The lateral pallial NSCs and neurons (blue) derive from few NE “progenitor pool” (dark blue) which are first amplified and become neurogenic only from 5dpf onward and persist life-long. Both aNSC populations remain segregated in space.

See also FigureS7.

Figure 7


A
her4:GFP
WT
PCNA
GFP
GS
wnt3a
her9
DAPI
3mpf
B
Tc
NE cells
lateral RGCs
dorso-medial RGCs
her4
wnt3a
her9
D
L
M
V
3mpf
C
RG progenitor
NE progenitor
Pa-Sp boundary
Dorso-medial domain
Dorso-medial neurogenesis
Lateral domain
Lateral neurogenesis
ob: Olfactory bulb
e : Epiphysis
Tc : Tela-Choroïda
v : Ventricule
Sp: Sub-pallium
Lateral view
Dorsal view
Cross section
Proneural clusters
Progenitor pools
1dpf
2dpf
5dpf
15dpf
3mpf
Onset of lateral neurogenesis

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1.9 Supplementary materials

Supplemental data

Figure S1 : Faithful *Cre* expression in *her4*$^{+}$ pallial neurogenic progenitors at embryonic and larvae stages in the *her4:ERT2CreERT2* line, related to Figure 1.

(A) Schematized three-dimensional representations and cross-sections of the telencephalon at embryonic (2dpf) and adult (3mpf) stages highlighting the position and the anatomical structure of the pallial GZ (orange), located along the VZ and composed at both stages of RGCs (orange triangles to cell bodies, thin orange lines to radial processes). Red doted lines delimite the pallium-subpallium boundary and purple arrows indicate the progression of neurogenesis. Due to eversion, the telencephalic ventricle is delimited by an extended roof plate at embryonic stages, which evolves to generate the tela-choroïda covering the adult telencephalic ventricle (blue). The tela choroïda is attached to the lateral edge of the pallial VZ (green arrowhead). OB: Olfactory Bulb, OP: Olfactory placode; Tel: Telencephalon; Ha: habenula.

(B) Compared *her4* expression (green) with *cre, gsh2* and *tbr1* (red) (left, middle and right columns, respectively), revealed by double in situ hybridization (ISH) on frontal sections of the telencephalon (single plane) of 2dpf *her4:ERT2CreERT2* embryos counterstained with DAPI (grey). OP: Olfactory placode; Tel: Telencephalon.

(C) Lateral 3D representations of the embryonic telencephalon in the *her4:ERT2CreERT2* line at 2dpf, with *her4* expression (green) compared to *cre* and *gsh2* expressing progenitors (left panel, magenta and orange respectively) or with *cre* expression compared to *tbr1* expressing progenitors (middle panel, yellow dots). Righ panels: Lateral and dorsal 3D representations of *her4*-positive pallial progenitors (red) at 2dpf in the embryonic telencephalon of an *her4:ERT2CreERT2* embryo. The ventricle is highlighted in grey. The white asterisks surrounded by dotted lines indicate the location of the anterior commissure on the lateral view, otherwise the dotted lines indicate the telencephalic VZ.

cre expression is restricted to the embryonic *her4*-positive pallial progenitors and is excluded from the subpallium defined by *gsh2* and *her4* expression. *tbr1* is expressed in differentiated cells of the pallium. Its expression delimits ventrally the boundary between the pallium and the pre-optic area.

(D) Compared *her4* expression (green) with *cre* expression (red), revealed by double in situ hybridization (ISH) on mid-sagittal (i) and para-sagittal (ii) focal planes from a stack of the telencephalon in *her4:ERT2CreERT2* embryos at 5dpf, counterstained with DAPI (grey). Double white arrows highlight the differential expression of *her4* and *cre* in the subpallium.

her4 and *cre* expression are however strictly colocalized in the pallium at 5dpf, as they were at 2dpf (see (C)).

(E) Cross-section of the adult telencephalon in a control *her4*^{switch} fish treated with EtOH at 1dpf, focusing on the pallium and immunostained for the recombined reporter mCherry (red) and counterstained with DAPI (grey) (n=3).

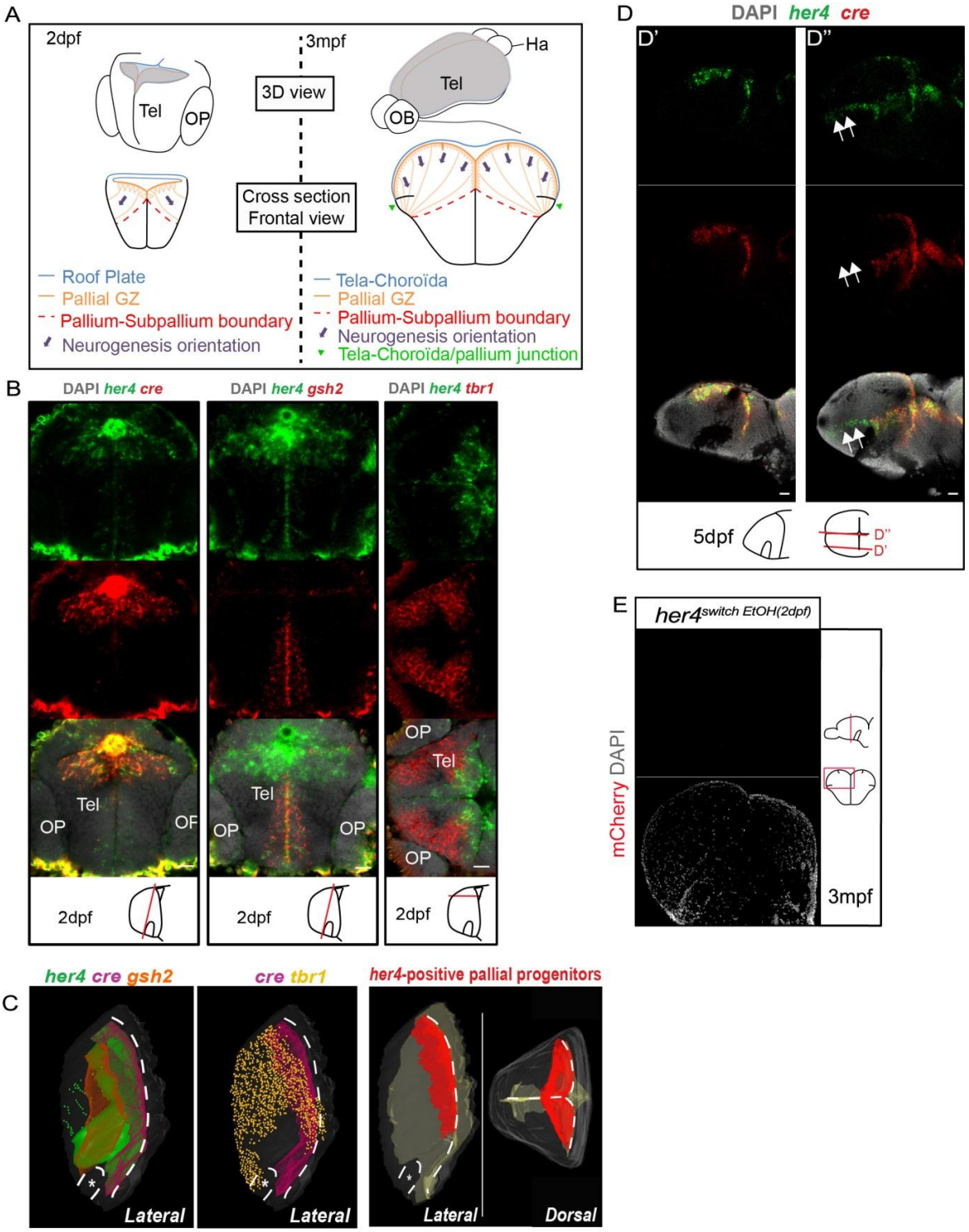


Figure S2: A large number of progenitors at 2dpf generates the dorso-medial aNSC population, related to Figure 2.

(A) Dorsal (upper panel) and lateral (lower panel) views of adult *ubi*$^{switch,T(2dpf)}$ telencephalon (whole-mounts showing one hemisphere, same clones as in panel 2B). The position of anterior and posterior section planes (panel S2B) are shown in magenta and blue.

(B) Cross-sections of the telencephali shown in A at the levels indicated (upper panel: magenta section plane; lower panel: blue section plane). The sections were stained for the recombined reporter mCherry (red) and the glial marker GS (green). Asterisks highlight clones in the medio-lateral pallium and lateral clones are numbered.

(C) Total number of RGCs (GS-positive/mCherry-positive cells) per dorso-medial or lateral clone (n=8 for dorso-medial clones and n=2 for lateral type1 and type2 clones). Values are presented as mean ± 95%CI (ANOVA).

(D) Medial cross-section of the telencephalon of an *hsp70*^{zebrabow,WoHS} adult whithout heat-shock treatment, focusing on the pallium. Note that the only reporter expressed is dTomato (no recombination).

(E) Dorsal whole-mount views of the dorso-medial domain of a *hsp70*$^{zebrabow,HS(2dpf)}$ adult (left column) showing expression of CFP/YFP (blue, green respectively, upper panel) or CFP/YFP/dTomato (blue, green and red respectively, lower panel). Cross-section of an adult *hsp70*$^{zebrabow,HS(2dpf)}$ telencephalon (right column), focusing on the dorsal pallium and showing expression of CFP/YFP (blue, green respectively, upper panel) or CFP/YFP/dTomato (blue, green and red respectively, lower panel). Note that small clones are very numerous and strongly intermingled. Because the brainbow reporter fish carry several copies of the transgene inserted on different chromosomes, the number of copies inherited by each animal, and the panel of colors produced, vary from fish to fish.

Figure S2

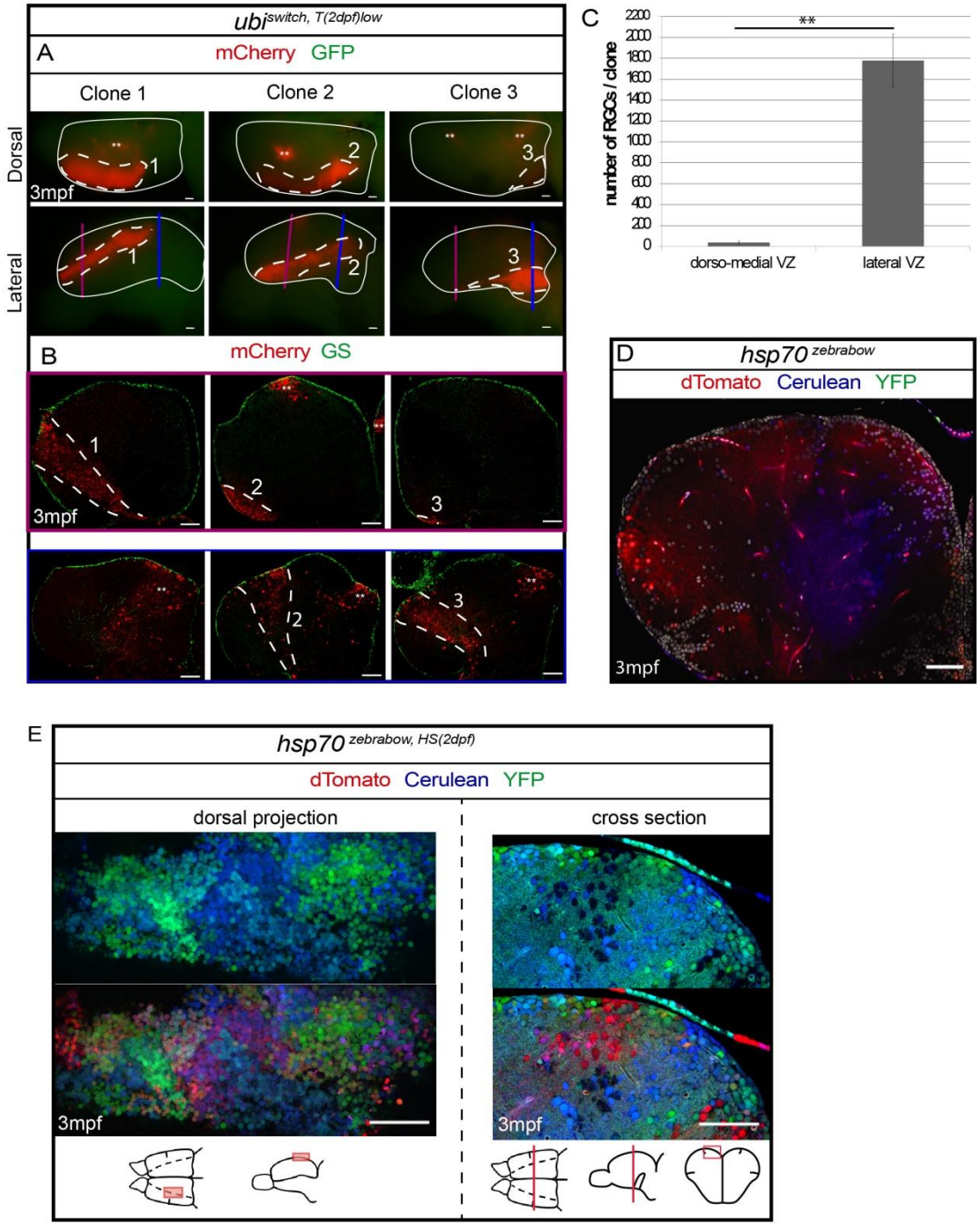

ubi switch, T(2dpf)low
A
mCherry GFP
Clone 1
Clone 2
Clone 3
Dorsal
Lateral
3mpf
B
mCherry GS
C
number of RGCs / clone
dorso-medial VZ
lateral VZ
D
hsp70 zebrabow
dTomato Cerulean YFP
E
hsp70 zebrabow, HS(2dpf)
dTomato Cerulean YFP
dorsal projection
cross section

Figure S3: The *her4*-negative embryonic territory fated to lateral aNSCs and present in the posterior telencephalic roof plate is laterally positioned after the eversion process, related to Figure 3.

(A) Frontal 3D views of the anterior prosencephalon, at 1dpf (upper panels), 1.5dpf (middle panels) and 2dpf (lower panels) stained with DAPI (grey). Right panels are magnifications of the red boxes showing the telencephalic dorsal midline after a 45°C rotation. The epiphysis is highlighted in pink, and the posterior telencephalic roof plate by asterisks. The eversion process results in the opening of the ventricle at 2dpf.

(B) *her4*-negative progenitors are present at the edge of the telencephalic VZ at 2dpf (upper panels) and 5dpf (lower panels). Cross-sections of *her4:GFP* telencephali, with magnifications of the boxed areas, immunostained for the reporter GFP (green) and the proliferating marker PCNA (red), and counterstained with DAPI (grey).

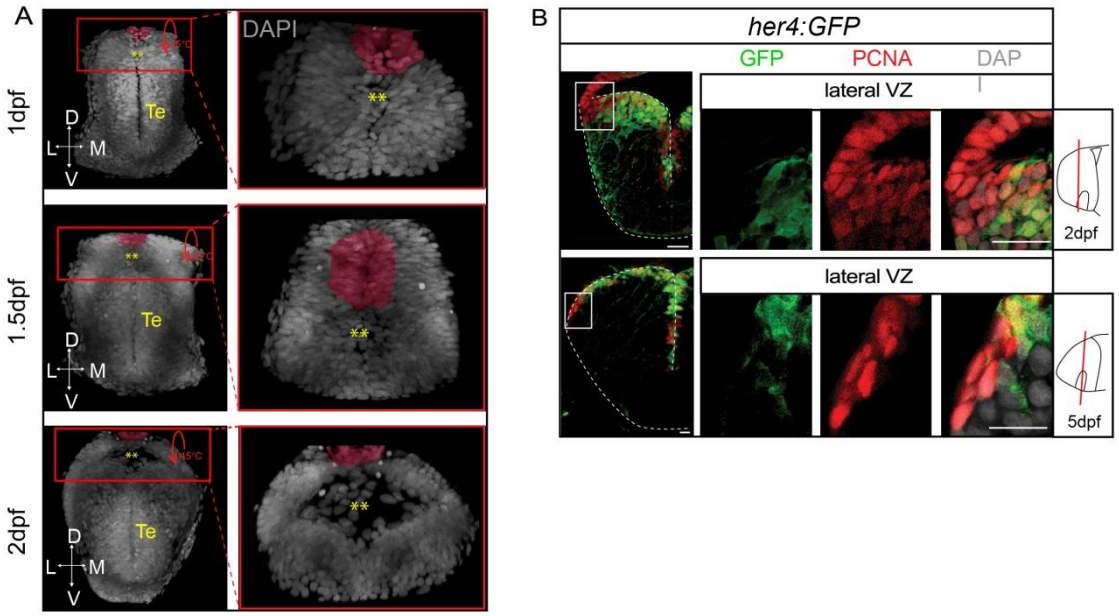


Figure S4: The progenitors at the origin of lateral pallial aNSCs are *her4*-negative throughout embryogenesis and express neuroepithelial progenitor markers, related to Figure 4.

(A) Experimental design to map the adult fate of cells expressing *her4* at the 1- to 10-somite stage (1ss-10ss) and at 1dpf, (B: *her4*$^{switch\text{T(1ss-10ss)}}$, C: *her4*$^{switch\text{T(1dpf)}}$) respectively.

(B,C) Cross-sections of the adult telencephalon in a control *her4*^{switch} fish treated with 4-OHT at 1ss-10ss (B) or at 1dpf (C), focusing on the pallium and immunostained for the recombined reporter mCherry (red) (DAPI counterstaining is shown in grey on the right panels) (n=3 brains each). The dorsal sulcus is indicated by the yellow arrowhead. Note that the limit of recombination obtained in B and C is identical to that observed when recombination is induced at 2dpf (Figures 1C, 5A).

(D) Cross-sections of the telencephalon in 1.5dpf embryos immunostained for GFP (green) in *gfap:GFP* fish (left panels), for the apical marker ZO1 (green) (middle panel), or for expression of the glial marker *blbp* (right panel, *in situ* hybridization, black) in *wt* fish, and counterstained with DAPI (grey or blue). Progenitors located at the posterior telencephalic roof plate (asterisks) are non-glial cells displaying an apico-basal polarity.

(E) Cross-sections of the telencephalon in 1.5dpf embryos immunostained for the neuronal marker HuC/D (blue) and either for GFP (green) in *her4:GFP* fish (left panels) or for the NSC markers Sox2 (red) (middle panel) and Musashi1 (red) (righ panel) in wt embryos, and counterstained with DAPI (grey). Progenitors located at the posterior telencephalic roof plate (asterisks) are GFP-negative and express Sox2 and Msi1.

Figure S4

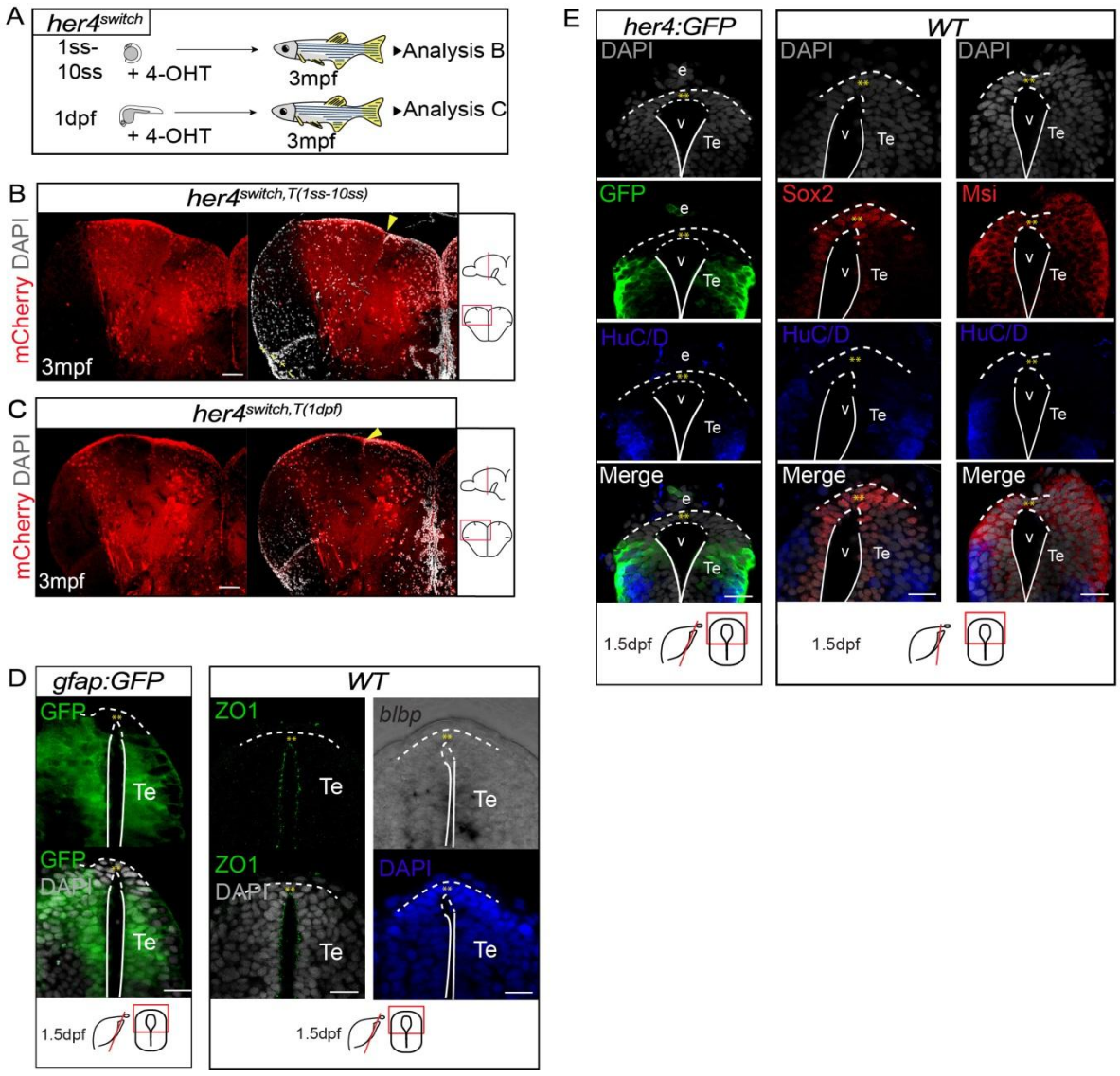

A
her4switch
1ss-10ss
+ 4-OHT
3mpf
Analysis B
1dpf
+ 4-OHT
3mpf
Analysis C
B
her4switch,T(1ss-10ss)
mCherry DAPI
3mpf
C
her4switch,T(1dpf)
mCherry DAPI
3mpf
D
gfap:GFP
GFP
GFP DAPI
Te
1.5dpf
WT
ZO1
ZO1 DAPI
blbp
DAPI
Te
1.5dpf
E
her4:GFP
WT
DAPI
GFP
HuC/D
Merge
Sox2
Msi
e
v
Te
1.5dpf

Figure S5: The number of lateral pallial progenitors increases after 5dpf concomitantly with neuron generation, related to Figure 5.

(A) Compared progression in the number of dorso-medial (PCNA-positive/mCherry-positive) and lateral (PCNA-positive/mCherry-negative) proliferating progenitors per telencephalic section at 5dpf, 15dpf and 1.5mpf (n=4 brains each, 3 sections per brain). Proliferating progenitors are mainly mCherry-positive at 5dpf (in average 35 mCherry-positive progenitors per section, representing 87% of VZ length in cell number, versus only 8 mCherry-negative progenitors, 13% of VZ length). The number of mCherry-negative progenitors massively increases thereafter (at 15dpf, 47 mCherry-negative progenitors are counted per section, representing 44% of VZ length). Values are presented as mean ± 95%CI.

(B) Compared percentage of proliferating progenitors in the dorso-medial (% of PCNA-positive/mCherry-positive progenitors among the total progenitors) and lateral (PCNA-positive/mCherry-negative progenitors among the total progenitors) VZ per telencephalic section at 5dpf, 15dpf and 1.5mpf (n=4 brains each, 3 sections analyzed per brain at equivalent levels). This percentage steadily decreases over time in the dorso-medial VZ starting at 5dpf while a maximum proliferation rate is maintained in the lateral VZ at least until late juvenile stages. Values are presented as mean ± 95%CI (ANOVA, **p<0.05).

(C) Experimental design for the analysis of lateral polyclones generated from embryonic progenitors in the lateral pallium at 2dpf (as in Figure 2A) and analyzed at larval (4dpf) (C') and juvenile stages (15dpf) (C"). (C') Cross section of a *ubi*$^{switch\ T(2dpf)}$ animal focusing on lateral polyclones at 4dpf (C') immunostained with the reporter marker mCherry (red), the proliferation marker PCNA (green) and the neuronal marker HuC/D (blue), and counterstained with DAPI (grey). Lower panels are magnifications of the boxed area. A lateral polyclone (mCherry-positive) is shown, comprised of a large progenitor population (PCNA-positive, surrounded by the dotted line) and a single neuron (Hu-positive, asterisk). (C") Cross section of a *ubi*$^{switch\ T(2dpf)}$ animal focusing on lateral polyclones at 15dpf (same immunostaining and symbols as in (C'), two polyclones are shown (numbered).

(D) Upper panel: Cross section of a *her4:GFP* telencephalon at 5dpf after a pulse of BrdU incorporation, immunostained for GFP (green), PCNA (red) and BrdU (blue), and counterstained with DAPI (grey). (D'-D") are high magnifications of the boxed area.

(E) Lower panel: Quantification of the labelling index (percentage of BrdU-positive cells within the PCNA-positive cells) in *her4*-positive or *her4*-negative progenitor populations in larvae as illustrated in (D). Values are presented as mean ± 95%CI (ANOVA).

Figure S5

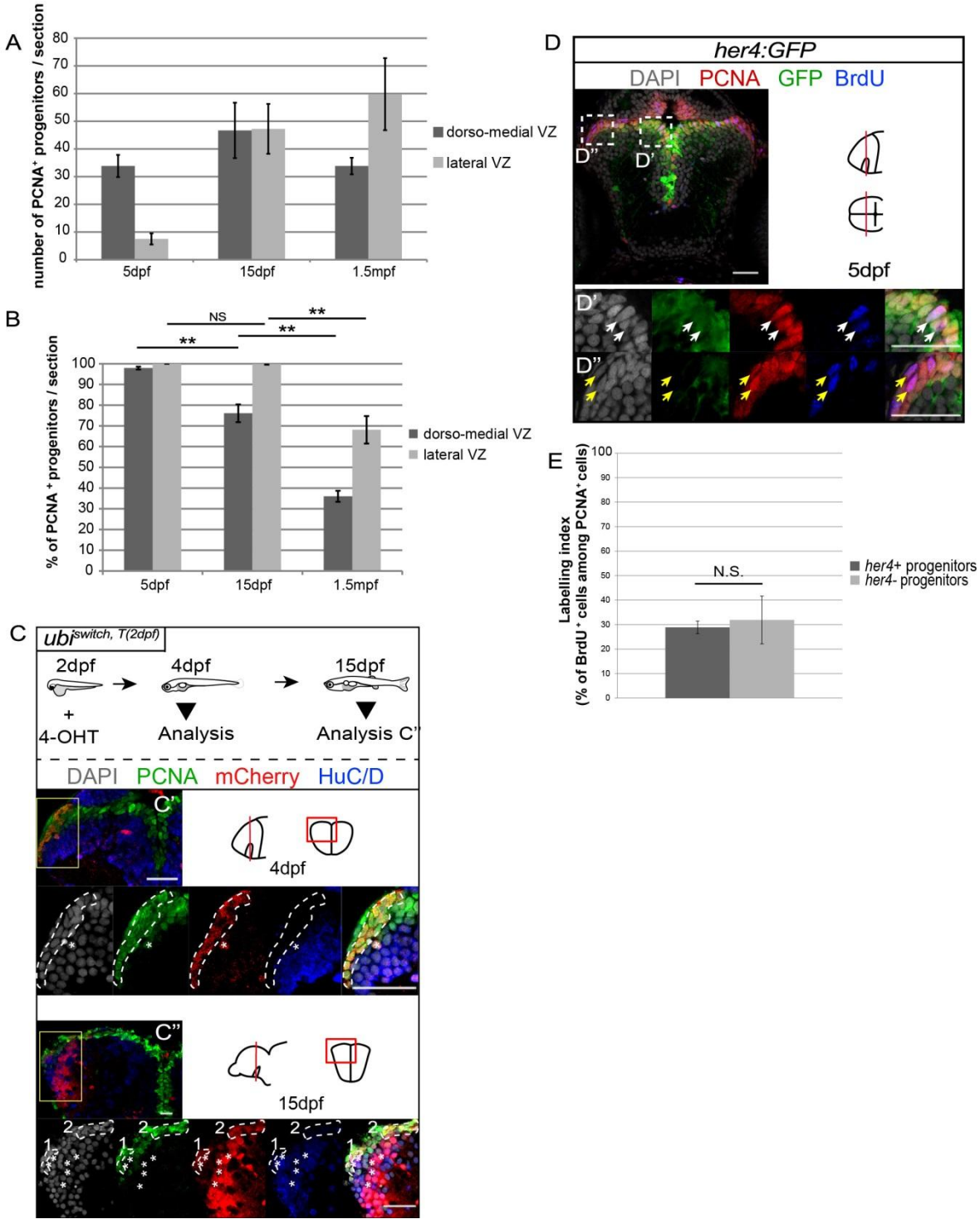

A
number of PCNA+ progenitors / section
80
70
60
50
40
30
20
10
0
5dpf
15dpf
1.5mpf
dorso-medial VZ
lateral VZ
B
% of PCNA+ progenitors / section
NS
**
**
**
100
90
80
70
60
50
40
30
20
10
0
5dpf
15dpf
1.5mpf
dorso-medial VZ
lateral VZ
C
ubiswitch, T(2dpf)
2dpf
+
4-OHT
4dpf
Analysis
15dpf
Analysis C'
DAPI PCNA mCherry HuC/D
C'
4dpf
C''
15dpf
1
2
D
her4:GFP
DAPI PCNA GFP BrdU
D''
D'
5dpf
D'
D''
E
Labelling index
(% of BrdU+ cells among PCNA+ cells)
100
90
80
70
60
50
40
30
20
10
0
N.S.
her4+ progenitors
her4- progenitors

Figure S6: Notch-insensitive NE cells remain at the pallial edge in the juvenile, related to Figure 6.

Cross-sections at posterior telencephalic levels in LY411575-treated fish, focusing on the lateral pallium and immunostained for PCNA (green), together with (red) the radial glia marker BLBP or the reporter mCherry in wildtype (upper panel) and *her4*$^{switchT(2dpf)}$ fish (lower panel), respectively. Both were counterstained with DAPI (grey). Arrowhead points to the small remaining pool of lateral Notch-insensitive progenitors.

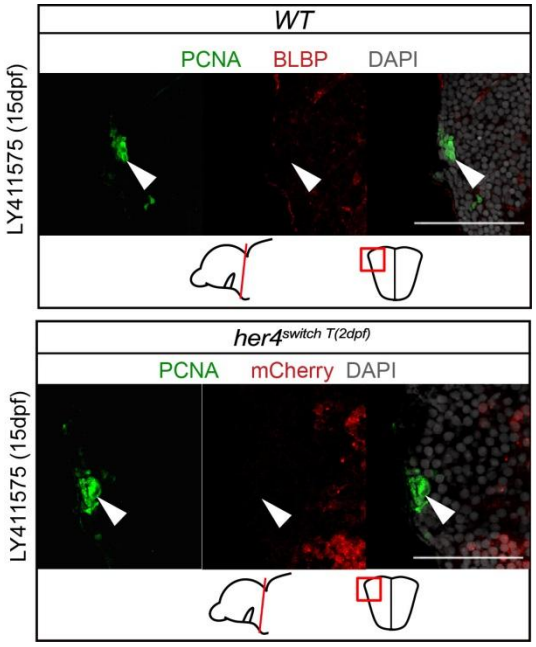


Figure S7: Some "progenitor pool"-like cells are consistently maintained at edge of the lateral pallial VZ until adult stage, related to Figure 7.

(A) Ventral view of adult telencephali labelled as whole-mount by ISH for *wnt3a* (left) or *her9* (right) expression. Dotted lines highlight the olfactory bulbs (OB) and orange arrows point to the postero-lateral "progenitor pool"-like population at 3mpf.

(B) Cross section of an adult telencephalon stained as whole-mount (upper right corner, lateral view) for *ascl1b* expression, focusing on the lateral pallium at posterior levels. Same indications as in (A).

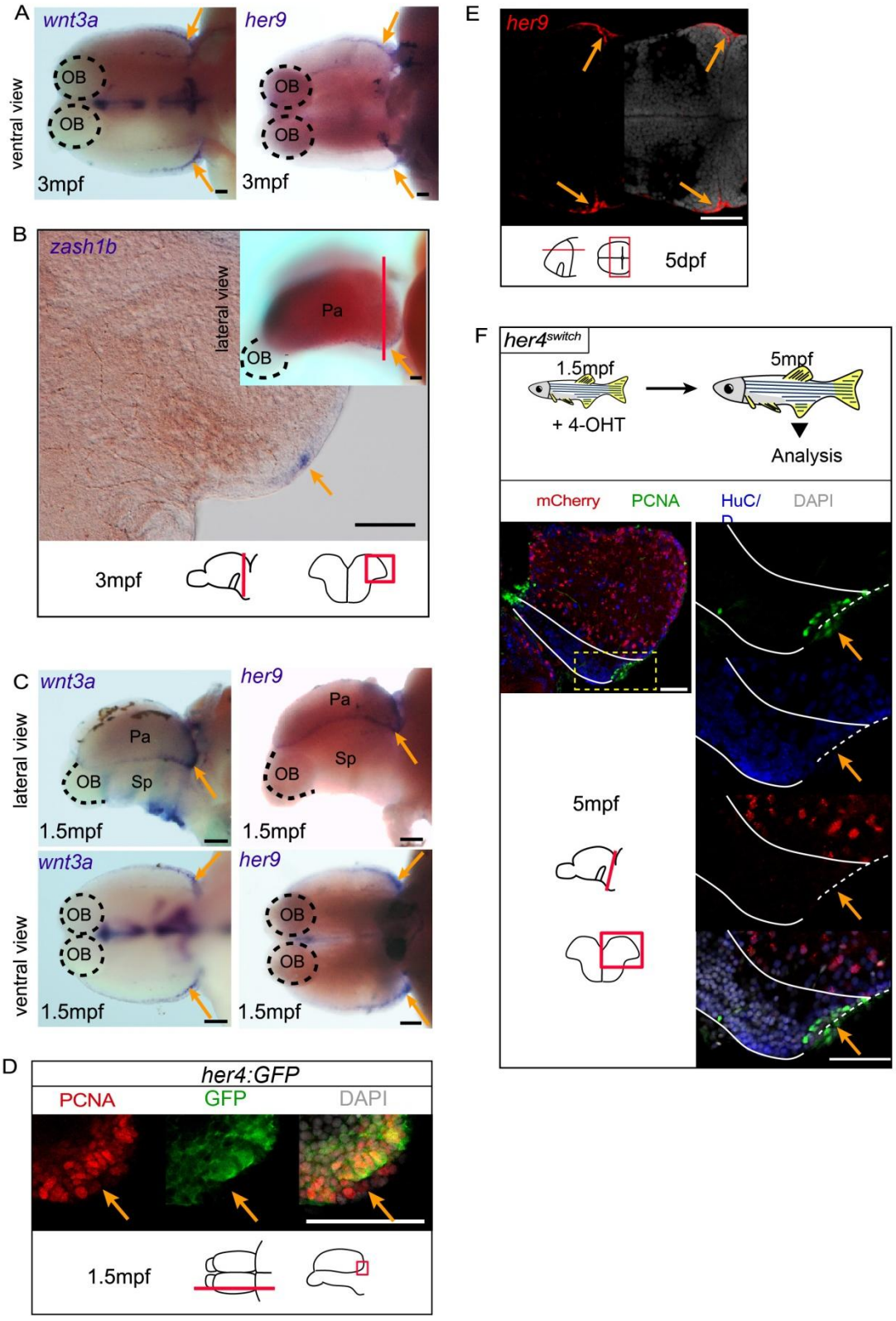
(C) Lateral (upper panel) and ventral (lower panels) views of telencephali at 1.5mpf labelled as whole-mount by ISH for *wnt3a* (left) or *her9* (right) expression. Arrows point to the postero-lateral "progenitor pool"-like population at 1.5mpf.

(D) Parasagittal section of a *her4:GFP* telencephalon at 1.5mpf, focusing on the lateral VZ at posterior levels, immunostained for GFP (green) and PCNA (red), and counterstained with DAPI (grey). Arrows point to the posterior *her4*-negative PCNA-positive population.

(E) Dorsal view (single plane from a confocal stack) of the telencephalon at 5dpf, focusing on the posterior part and showing *her9* expression (red) revealed by fluorescent ISH. Arrows point to the postero-lateral "progenitor pool"-like population at 5dpf.

(F) Experimental design for the analysis of the adult fate of progenitors expressing (or not) *her4* at 1.5mpf using *her4*^{switch} fish (upper panel): fish were treated at 1.5mpf with 4-OHT and analysed after a long chase of 3.5 months. Cross-section of adult telencephalon of *her4*$^{switchT(1.5mpf)}$ fish at 5mpf, focusing on the posterior pallium and stained with mCherry (red), PCNA (green), HuC/D (blue) and DAPI (grey). Dotted lines on high magnification panels delimit the lateral edge of the postero-lateral VZ, and white lines the mCherry-negative territory. Arrows point to the postero-lateral "progenitor pool"-like population at 5mpf.

Figure S7


A
wnt3a
her9
ventral view
OB
OB
3mpf
3mpf
B
zash1b
lateral view
Pa
OB
3mpf
C
wnt3a
her9
lateral view
Pa
OB
Sp
1.5mpf
1.5mpf
wnt3a
her9
ventral view
OB
OB
1.5mpf
1.5mpf
D
her4:GFP
PCNA
GFP
DAPI
1.5mpf
E
her9
5dpf
F
her4switch
1.5mpf
+ 4-OHT
5mpf
Analysis
mCherry
PCNA
HuC/
DAPI
5mpf

Supplemental Experimental Procedures

4-OHT treatments

RECOMBINATION STAGE	4-OHT CONCENTRATION	TIME
5ss-10ss	10µM	2h
24hpf - 48hpf	10µM	5h
3dpf	10µM	24h
5dpf-6dpf	10µM	30h
15dpf -20dpf	Day1: 7.5µM	24h
	Day2: 5µM	24h
	Day3: 7.5µM	24h
	Day4: 5µM	24h
1.5mpf	5µM	72h

Table for the conditions of 4-OHT treatments

For treatments at 15dpf, *her4*^{switch} fish were placed in fresh 4-OHT solution every 24 hours. At 1.5mpf, 4-OHT solution was added for only 9h/day, thus alternating treatment and recovery periods.

Immunohistochemistry and *In Situ* Hybridization

antibodies	*Species*	*Dilution*	*ref*
anti-HuC/D	human	1/2000	Dr B.Zalc
anti-glutamine-synthase	mouse	1/500	Millipore, MAB302
anti-PCNA	mouse	1/250	Santa Cruz, PC-10
anti-mCherry	rabbit	1/300	Clontech
anti-GFP	chicken	1/1000	Avec.Lab.
anti-sox2	rabbit	1/500	abcam, Ab97959
anti-ZO1	mouse	1/200	life techno.
anti-musashi1a	rabbit	1/200	Sakakibara et al., 1996

Table of antibodies

Goat antibodies coupled to AlexaFluor dyes (488, 555, or 647; Invitrogen) and DAPI were used as secondary antibodies and nuclear staining. *In toto* immunostaining on embryos were performed following 3 hours of sweet PFA fixation (4% PFA-4% w/v sucrose in PBS).

GENE	PROBE CONCENTRATION	PROBE REFERENCE
her4.1	0,5ng/µl FL coupled	Takke et al., 1999
ert2Cre	0,78ng/µl Dig coupled	PCR amplification
gsh2	0,2ng/µl Dig coupled	
tbr1	1,25ng/µl Dig coupled	Mione et al. 2001
wnt3a	0.4ng/µl, Dig-coupled	Mattes et al., 2012
wnt8b	0.3ng/µl, Dig-coupled	kelly et al., 1995
fgf8	0.5ng/µl, Dig-coupled	Topp et al., 2008
bmp6	0.5ng/µl, Dig coupled	SourceBioscience, BC090689
her6	0,2ng/µl Dig coupled	SourceBioscience, BC059551
her9	80ng/µl, Dig coupled	SourceBioscience, BC079516
blbp	78ng/µl, Dig coupled	Liu et al., 2003
zash1b	60ng/µl, Dig coupled	Allende et al., 1994

Table of ISH probes

The *Ert2Cre* sequence was amplified by PCR from *pCDNA:Ert2CreErt2* (Jullien, N. et al., 2008) (primer sequences Ert2-fw ATGGCCGGTGACATGAGAGCTG, Cre-rv CATCAGGTTCTTCCTGACTTCAT) and subcloned into pCSA (Clontech).

Supplemental References

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Takke, C., Dornseifer, P., Weizsäcker, von, E., and Campos-Ortega, J.A. (1999). her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling. *126*, 1811–1821.

2 The successive maturation steps from the embryonic neural progenitor to the adult NSC

In this chapter, I will consider my previous results (results – section1) in a different context, and detail some additional experiments, to further ask what the different maturation steps are that progressively bring from embryonic neural progenitors to adult NSCs.

It is difficult to directly answer this question. Indeed, we are not able to follow and precisely characterize small groups of progenitors over a very long time period. Thus, at this point, data in the literature mainly focused on the temporal evolution of progenitors during neurogenesis in the embryo. In the mouse, it is currently accepted that early NE cells are replaced with time by RGCs. There, embryonic progenitors are classified according to four criteria: (i) their morphology (NE or RGC), (ii) their Notch sensitivity, (iii) their expression of *Hes/her* genes and finally (iv) their neurogenic capacity.

To know whether progenitors always mature following the same sequence of events, in particular to generate aNSCs, I have thus started to use the four criteria worked out in the embryo, to challenge dorsal and lateral progenitor subtypes at different stages between embryonic stage and adult stage (ie. 5 dpf, 1 mpf, adult).

In a first short part below, I will report on a potential Notch-independent maintenance of dorsal pallial progenitors. Then, I will focus on lateral progenitors. In the *Dev Cell* publication, we have shown that NE cells can persist at adult stage and I was able to distinguish in the adult lateral pallium a series of progenitor subtypes progressively giving rise to mature aNSCs (the *her9*-positive NE cells, the Notch sensitive-NE cells, the *her4*-positive NE cells and *her4*-positive GS-positive RGCs, which would represent progressive maturing steps from the lateral edge of the VZ toward the medial pallium). This recapitulates in space what might be the maturation steps of progenitors over time. Together, this shows first that NE progenitors are not all transformed into RGCs, and second provides information on the different steps of progenitor maturation, which I will compare with the embryonic sequence.

2.1 Introduction

During development, progenitors composing the brain are submitted to different changes regarding morphology, neurogenic capacity, or gene expression. The neural plate is composed of NE cells induced from the ectoderm by neural promoting signals (Andoniadou and Martinez-Barbera, 2013). These NE cells, after several rounds of symmetric divisions to amplify the population (Götz and Huttner, 2005), start to generate neurons. Under the influence of certain factors, such as Pax6 in the mammalian brain (Suter et al., 2009), they further progressively generate a second type of progenitors, the RGCs, performing mainly

asymmetrical neurogenic divisions to generate neurons (Kriegstein and Alvarez-Buylla, 2009). This process has been extensively studied in the developing mammalian cortex in which the transition between NE cells and RGCs occurs between E10.5 and E16.5 with a antero-lateral to postero-medial gradient (Anthony et al., 2004). During mouse brain development, the RGCs generate neurons and other glial cells such as astrocytes, ependymal cells and oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009), but also aNSCs (Anthony et al., 2004; Malatesta et al., 2003). However, these studies were done either at an early stage or using non-conditional lineage tracing experiments with an analysis in the adult. This leads to a model in which one type of progenitor replaces the other, but this view might be incomplete. Indeed, these different progenitors might coexist at a particular time point but also persist at later stages. Moreover, whether the series of maturation steps, in particularly concerning its molecular sequence, is conserved between the progenitors at the origin of aNSCs remains to be determined.

One of the main signals involved in controlling, on the one hand embryonic neural progenitors, and on the other hand aNSCs, is the Notch pathway (Bae et al., 2005; Chapouton et al., 2010; Imayoshi et al., 2010; Takke et al., 1999). In neural progenitors, the main Notch target are the *hairy and enhancer of split* related genes (*Hes* in mammals and *her* in the zebrafish). In mouse, *Hes* mutants display a premature progenitor differentiation (Hatakeyama and Kageyama, 2006; Hatakeyama et al., 2004). Similarly, in the zebrafish embryo, the so-called canonical *her* genes, such as *her4* (homologous to *Hes5* gene) (Takke et al., 1999), are activated by the Notch pathway and maintain the embryonic neurogenic progenitors (Stigloher et al., 2008).

On the contrary, the progenitor pools, such as the embryonic NE cells located at neural tube boundaries, express non-canonical *her* genes such as *her6*, *her9* or *her5*. These genes expression is independent of Notch signalling but is activated by positional cues responsible for patterning the neural plate (Bae et al., 2005; Geling et al., 2003, 2004; Hans et al., 2004). In the early embryo, the non-canonical *her* genes are also necessary to maintain progenitors. Specifically, they are involved in delaying the neurogenic activity of these embryonic progenitors and their transition toward actively neurogenic proneural clusters (Stigloher et al., 2008). Indeed, downregulating non-canonical *her* genes transforms progenitor pools into neurogenic proneural clusters (Geling et al., 2004; Scholpp et al., 2009). In both mouse and zebrafish, *Hes*/*her* genes are expressed in both embryonic progenitor subtypes (Hatakeyama et al., 2004; Stigloher et al., 2008) and aNSCs (Chapouton et al., 2011; Lugert et al., 2010; Stump et al., 2002). However, the relationship between *Hes*/*her* expression, the transition between NE and RGCs, and the neurogenic activity, as well as the role of these genes later during progenitor maturation remain to be determined in both species.

In the zebrafish, I demonstrated that the adult pallial germinal zone derives from the two different progenitor populations: the embryonic “proneural clusters” composed of neurogenic active progenitors that amplify throughout life to generate aNSCs of the dorso-medial pallium, and the NE “progenitor pools” located in the telencephalic roof plate at early stage. These NE cells contribute from juvenile stage onward including at adult stage to the generation of aNSCs of the lateral pallium (see *Dev Cell* publication – section 1). These two different modes of stem cells formation, physically segregated in the zebrafish pallium, raise several questions: (i) what are the steps involved in the emergence of pallial NSCs from embryonic progenitors, (ii) what are the signals involved in maintaining long-lasting neural progenitors, (iii) are these parameters comparable in the medial and lateral pallium, and (iv) how do they compare with the “classical” sequence known for progenitors during embryonic neurogenesis, described above.

To start addressing these issues using the zebrafish pallium as a model, I will consider, in the dorsal pallium, the Notch-sensitivity of the embryonic and juvenile neural progenitors in link with progenitor maintenance and *her4* expression (see *Dev Cell* publication – section 1). Next, by analyzing the late juvenile and adult lateral VZ, a site of continuous aNSCs formation in the zebrafish telencephalon (see *Dev Cell* publication – section 1), I will provide an overview of the different steps involved in progenitor maturation, and determine whether adult and embryonic processes are similars to generate RGCs.

2.2 Results

2.2.1 Heterogeneity of the dorso-medial progenitors regarding Notch maintenance at 1dpf

Lineage tracing experiments of *her4*-positive embryonic progenitors of the zebrafish pallium indicate that, a some point during development, all the progenitors at the origin of the aNSCs of the zebrafish pallium express *her4* (see *Dev Cell* publication – section1.4.6). Moreover, Notch inhibition at 2dpf as well as 15dpf indicates that *her4*-positive cells are maintained by the Notch pathway, at least from 2dpf onward (see *Dev Cell* publication – section 1.4.4 and 1.4.6). However, it remains unclear whether *her4* expression and Notch-dependent maintenance are always correlated in neural progenitors during development.

We thus investigated whether Notch is required for the maintenance of *her4*-positive RGCs before 2dpf. To do so, I treated *her4:ERT2CreERT2;ubi:switch* double transgenic embryos with 4-OHT for 2 hours at 1dpf. Then, I treated the embryos with LY411575 for 6 hours at the high concentration of 50µM, and analysed the results at 5dpf (Figure 30A).

Interestingly, contrary to the massive depletion of *her4*-positive progenitors after a LY411575 treatment at 2dpf (see *Dev Cell* publication, Figure 4E-G), only a subset of the progeny of the *her4*-positive cells at 1dpf is depleted at 5dpf after a LY411575 treatment at 1dpf (Figure 30A). Indeed, compared to the control situation in which the ventricular zone is composed of proliferating cells, the absence of PCNA-positive cells in LY411575-treated fish was confined to the most medial part of the pallium, suggesting that only progenitors in this region differentiated (Figure 30A). This is in accordance with the results obtained with a similar treatment at 2dpf, indicating that at both 1dpf and 2dpf, Notch signaling maintains the most medial part of the embryonic ventricular zone. However, we noticed that a population of mCherry-positive/PCNA-positive cells is still present dorsally (Figure 30A – white star), adjacent to the mCherry-negative progenitors (that will later give rise to the lateral aNSCs population (see *Dev Cell* publication - section 1). The same LY411575 treatment on *her4:GFP* embryos indicates that some of the PCNA-positive cells maintained at the ventricular zone express *her4* at 5dpf (Figure 30B – yellow arrow), and treating *gfap:GFP* embryos further shows that some of them are glial cells as they express GFP (Figure 30C – magenta arrows). These results could indicate that whithin the *her4*-positive telencephalic progenitors at 1dpf, the most medial *her4*-positive progenitors are maintained by the Notch pathway, whereas the most dorsal *her4*-positive progenitors are maintained independently of Notch signaling.

These results highlight that, early during development, it might exist an heterogeneity within the dorso-medial RGCs cells regarding the implication of the Notch pathway on progenitor maintenance.

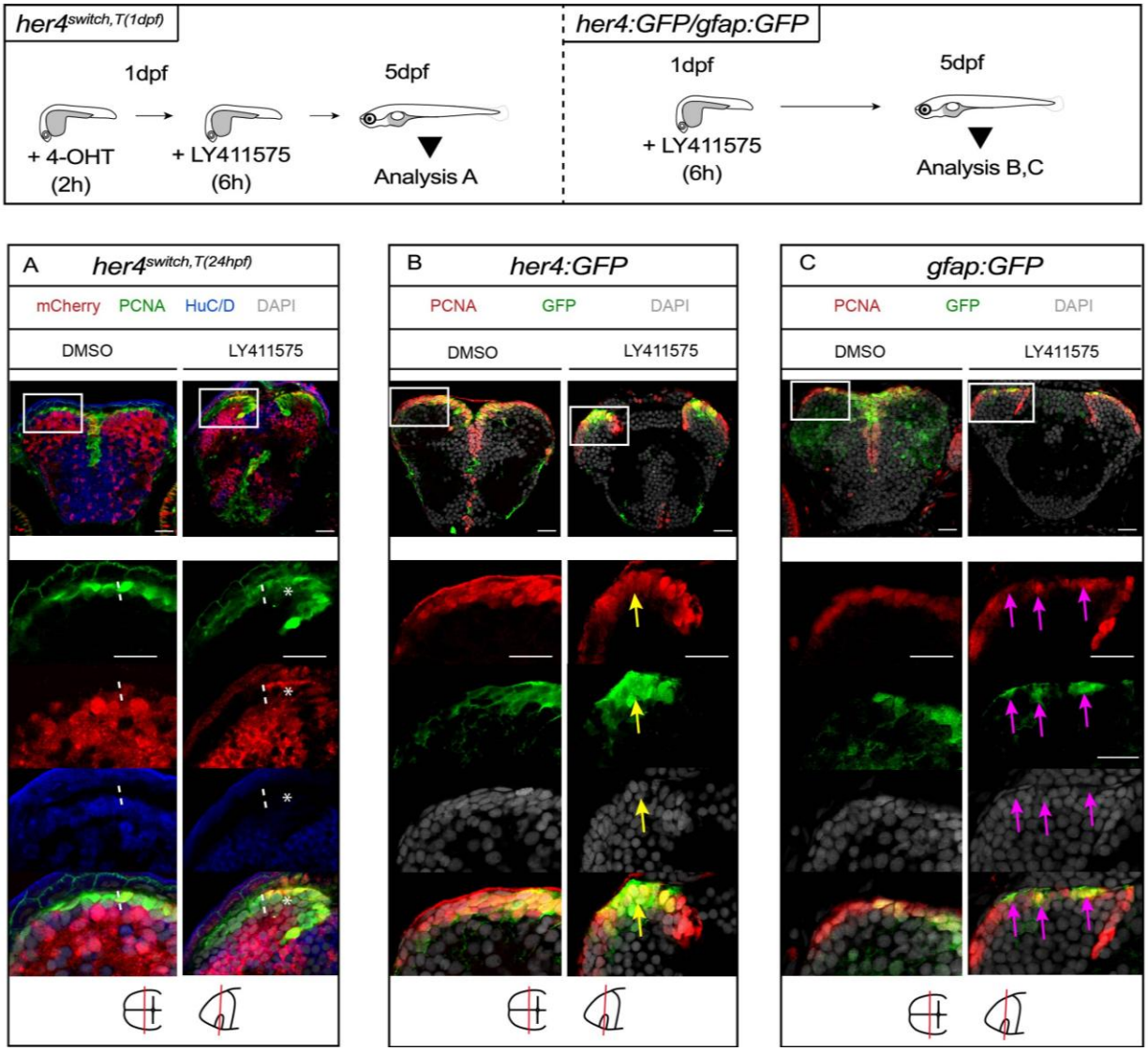


Figure 30: Only the most medial pallial progenitor population may be maintained by the Notch pathway at 1dpf

(A) Medial cross-sections of the telencephalon in her4$^{switchT(1dpf)}$ larvae at 5dpf treated with DMSO or LY411575 with magnification of the dorso-lateral VZ, immunostained as indicated. The white dotted line highlights the dorso-medial/lateral pallial boundary, and the white star the dorsal mCherry-positive/PCNA-positive cells after LY411575 treatment at 1dpf.

(B) Medial cross-sections of the telencephalon of her4:GFP larvae at 5dpf treated with DMSO or LY411575 at 1dpf with magnification of the dorso-lateral VZ, immunostained as indicated. The yellow arrows indicate the dorsal GFP-positive/PCNA-positive cells after LY411575 treatment at 1dpf.

(C) Medial cross-sections of the telencephalon of gfap:GFP larvae at 5dpf treated with DMSO or LY411575 at 1dpf with magnification of the dorso-lateral VZ, immunostained as indicated. The magenta arrows indicate the dorsal GFP-positive/PCNA-positive cells after LY411575 treatment at 1dpf.

2.2.2 Different progenitors at the ventricular zone reflect distinct maturation states

We have shown in the *Dev Cell* publication that during development NE "progenitor pool" cells (Notch-independent and *her4*-negative) progressively generate lateral pallial aNSCs. Indeed, once induced, lateral RGCs display common features with the dorso-medial population – ie. *her4*-positivity and Notch-sensitivity- (Chapouton et al., 2010; März et al., 2010b). These findings indicate that there is a maturation gradient of the embryonic NE progenitors toward the "aNSC state" and this seems to go through an activation of *her4* expression and the acquirement of the RG phenotype. In addition, we observed that a small population with "progenitor pool" features remains present all along life and participates in the generation of *de novo* aNSCs in the most postero-lateral part of the adult pallium (see *Dev Cell* publication – section 1.4.7). Thus, the lateral pallial VZ seems to be a good model to study the different steps necessary for the generation of mature aNSCs. We thus investigated the different progenitors present within the lateral population at 5dpf, 1mpf and 3mpf, with the aim of getting a first overview of the different types of progenitors present at the VZ at different stages of late development.

- *Progenitor composition of the lateral pallial VZ at 5dpf*

In order to precisely determine the location of RG cells at the larval stage and confirm that they are absent from the lateral progenitor domain, I first analysed the expression of the GS marker at 5dpf in *her4:ERT2CreERT2;ubi:switch* double transgenic embryos recombined at 1dpf. GS is not expressed in the mCherry-negative population (Figure 31A – white stars), indicating that only the dorso-medial progenitors display RG features. At 5dpf, all lateral neural progenitors would thus correspond to NE cells. Interestingly, we demonstrated that at this stage, *her4*-expression is already induced in the most dorsal part of the lateral VZ (see *Dev Cell* publication – section 1.4.6), thus indicating that already at 5dpf, the lateral progenitor population is heterogenous with GS-negative/*her4*-positive progenitors close to the dorso-medial population and GS-negative/*her4*-negative progenitors at the edge of the VZ.

- *Progenitor composition of the lateral pallial VZ at 1mpf and 6mpf*

As *her4*-positive RG cells are generated later than 5dpf in the lateral VZ, we analysed precisely *her4* expression at the postero-lateral edge of the late juvenile and adult VZ using *her4:GFP* fish. We already demonstrated that in the region of the adult pallium, NE cells are present at the boundary between the tela-choroïda and the lateral VZ and this population would be responsible for generating *her4*-positive RGCs (see *Dev Cell* publication – section

1.4.7). Interestingly, comparison of GS and GFP expression in the *her4:GFP* juvenile at 1mpf and 3 months indicate that a *her4*-positive/GS-negative population is always present abuted to the RGCs (Figure 31B – yellow star and line), and located between the NE “progenitor pool” cells (PCNA-positive/*her4*-negative/GS-negative) (Figure 31B – magenta star) and the activated RGCs (PCNA-positive/*her4*-positive/GS-positive populations) (Figure 31B – white star and line).

Altogether, these results highlight that, at all juvenile and adult developmental stages, the same potential gradient of maturation necessary for the generation of aNSCs is present at the postero-lateral edge of the pallial VZ: first, NE “progenitor pool” cells expressing *her6* and/or *her9* (see *Dev Cell* publication – section 1.4.4), then, *her4*-positive NE cells, and finally *her4*-positive RGCs that will become mainly quiescent (Chapouton et al., 2010; März et al., 2010b).

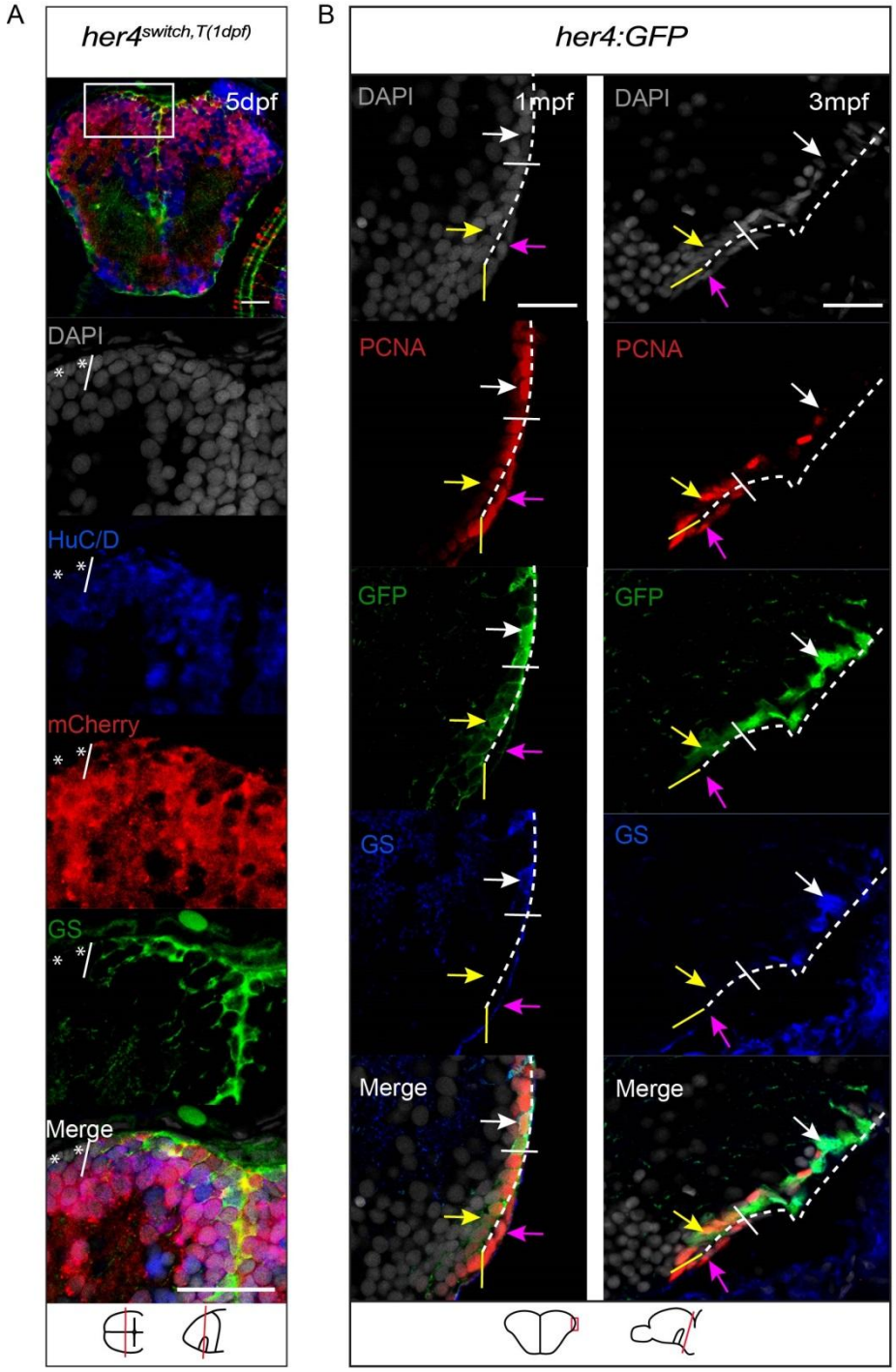


Figure 31: Several types of progenitors reflecting different maturation states are present at the postero-lateral edge of the pallial VZ

(A) Medial cross-sections of the telencephalon in her4:switch$^{T(1dpf)}$ larvae at 5dpf, immunostained as indicated. White dotted line highlights the dorso-medial/lateral pallial boundary, and white stars the GS-negative/mCherry-negative/HuC/D-negative cells.

(B) Posterior cross sections focusing on the lateral edge of the juvenile (left panel) and adult (right panels) VZ in her4:GFP fish immunostained as indicated. The white arrow and line highlight the GS-positive/her4-positive population. The yellow arrow and line highlight the GS-negative/her4-positive population. The magenta arrow indicates the GS-negative/her4-negative cells. The white dotted line highlights the ventricular zone.

2.3 Conclusion and preliminary discussion

In this section, I addressed the question of the maturation of the neural progenitors at the origin of aNSCs. For this, I both reanalyzed existing data (Dev Cell article) and added new experiments, which together suggest that (i) differences may exist between the dorso-medial and the lateral VZ regarding the acquisition of a Notch-dependent maintenance, and (ii) in the lateral VZ, a gradient of progenitor maturation takes place with different steps running from the NE "progenitor pools" to the *her4*-positive RGCs through a NE *her4*-positive state (Figure 32).

- *The maintenance of the dorsal RGCs might be Notch-independent*

The Notch inhibition experiments reported here suggest that, among the *her4*-positive embryonic population at the origin of the dorso-medial VZ, the most dorsal progenitors display a Notch-independent phase at early larval stages, whereas the most medial VZ is depleted upon Notch inhibition. Similar treatments at 2dpf lead to different results with a massive depletion of the entire *her4*-positive population (see *Dev Cell* publication – Figure 4F). Thus, changes in Notch dependency may take place in dorsal progenitors between these two stages. In addition, we already demonstrated that Notch inhibition at 15dpf, leads to the depletion of both medial and dorsal progenitors (see *Dev Cell* publication – Figure 6C). Altogether, these results suggest that dorsal progenitors become Notch-dependent from 2dpf onward, whereas the medial population is maintained by the Notch pathway from at least 1dpf.

It has already been shown that *her4* expression is regulated by Notch in the early embryo as overexpression of NICD leads to ectopic *her4* expression (Takke et al., 1999). In our experiment, the differential Notch dependence of the *her4*-positive progenitors raises the question of the potential regulation of *her4* expression by other signaling pathways than Notch at very early embryonic stages. In the mouse, *Hes5* expression has always been associated with the Notch pathway and is considered as the most faithfull read-out of Notch activity. It was shown that, very early during embryonic development, some *Hes* genes start to be expressed independently of Notch signaling , but this particular regulation was reported only for *Hes3* and *Hes1*, while *Hes5* expression was associated with the expression of Notch signaling components (Hatakeyama and Kageyama, 2006). However, whether the initiation of *Hes5* expression in the neural plate was truly dependent on Notch signaling was not directy tested. In zebrafish, similar Notch independent regulation of non-canonical *her* genes has been reported, eg. for *her3* (homologous to the mammalian *Hes3*), *her5* (Geling et al., 2003) and *her9* (homologous to the mammalian *Hes4*) (Bae et al., 2005; Hans et al., 2004). Concerning *her4* expression, no particular Notch-independent regulation was reported so far.

her4 expression is initiated at 70% epiboly and further extends to different “proneural cluster” domains within the neural plate (Takke et al., 1999). The functional evidence for *her4* being a Notch target relies on electroporation experiments of NICD that activate ectopic *her4* expression at the neural plate stage (Takke et al., 1999). However, while this demonstrates that Notch signaling is sufficient to activate *her4*, it does not show that it is necessary for its initiation in the neural plate. My observations constitute only preliminary results and much work remains to be done. First, we would need to analyse *her4* expression at around 30hpf, the end of the LY treatment performed at 1dpf; if *her4* is expressed in the dorsal progenitors at the end of the treatment, it would confirm that its expression could be independent of Notch signalling and the signals involved in maintaining the dorsal progenitors could go through the Her4 factor. In this case, the specific inhibition of *her4* expression would confirm whether or not Her4 is itself responsible for maintaining this population. Moreover, it could be interesting to investigate the dynamics of Notch pathway components expression in the dorso-medial region and determine whether a subpopulation of the dorsal-medial region does not express any Notch3 or Notch1 receptors at 1dpf even though *her4* is present in the population; the latter would emphasize the presence of Notch-independent *her4* expression.

Second, it would be interesting to investigate whether, as for the “boundary” populations, some dorso-medial progenitors are maintained by positional cues. We can notice that the dorsal progenitors is the population abuted to the roof plate in the very early embryo (at 1dpf), we could thus hypothesize that some signals coming from this region are responsible from maintaining the dorsal progenitors. By the growth of the pallium and the eversion process, the distance between this population and the roof plate deriving cells increase and this could trigger the appearance of Notch sensitivity in the dorsal population. Whether BMP, FGF or Wnt signaling, present in the roof plate, play a role in maintaining the dorsal pallial progenitors and/or in initiating *her4* expression in this population would be interesting to test.

If *her4* is not involved in maintaining the dorsal population, we could also address whether other Notch-independent *her* genes are expressed specifically in the dorsal progenitors. We already know that *her6* and *her9* are not expressed in this population as they are present only in the roof plate (see *Dev Cell* publication – Figure 4B), and *her5* is only expressed at the midbrain-hindbrain boundary at 1dpf (Geling et al., 2003, 2004). *her15* has been shown to be expressed in the neurogenic progenitors but its expression is dependent on Notch signaling (Bae et al., 2005); but we could investigate whether *her12* or *her2,* normally expressed in proneural clusters, could be differentially expressed between dorsal and medial populations, as their Notch-dependency has not been clearly demonstrated yet.

Then, whether the presence of Notch-independent *her4* expression can be generalized to the medial progenitors remains to be addressed. We did not observe any *her4*-positive progenitors maintained independently of the Notch pathway when we performed the

treatement at 1dpf; however, this progenitor state could be present earlier during development. Thus, the experimental inhibition of Notch signaling should be perfomed earlier than 1dpf to address this point. If we consider this possibility, it would mean that the Notch-dependent maintenance is generally acquired after *her4* expression in dorso-medial progenitors (Figure 32).

Finally, NE progenitors compose the very early neural plate and the stage at which the transition toward RG cells occurs in the dorso-medial presumptive domain remains unclear. Indeed, we do not report the presence of NE cells in the dorso-medial population from 1dpf onward. Analysis of the glial markers by taking advantage of the *gfap:gfp* fish line or by performing *in situ* hybridization for *blbp* at 1.5dpf, indicate that both markers are expressed in the embryonic pallium and excluded from the roof plate. But it is interesting to note that contrary to *gfp* expression in the *gfap:gfp* line which is broadly expressed in the dorsal telencephalon, *blbp* seems to be restricted to the most medial population and its expression is not as large as *gfap* (see *Dev Cell* publication – Figure S4D). This suggests that within the dorso-medial domain, the heterogeneity whithin the glial population could reflect different maturation states also in this population, and highlight that even though all the progenitors have already undergone their transition toward a glial state at that stage, the medial and the dorsal population remain different. This could be reminiscent of the progressive acquisition of glial features reported in the mouse pallium (Anthony et al., 2004). A more acute analysis of the morphology and the different glial markers expression, as well as *her4* expression, could help us to determine when both dorsal and medial populations acquire glial features and whether this is correlated with the appearance of *her4* expression in the dorso-medial population.

In the lateral domain, which is formed late during development, we observed that lateral progenitors also become Notch-dependent. However, when we performed the LY411575 treatment at 15dpf, *her4*-positive and *her4*-negative ventricular cells were depleted except a minute population of NE cells at the postero-lateral edge of the pallial VZ. This means that contrary to the dorso-medial VZ of the early embryo, both *her4*-positive and some *her4*-negative lateral progenitors are maintained by the Notch pathway; indicating that the Notch-dependent maintenance may be acquired before *her4* expression in these lateral progenitors. This highlights that the lateral and dorso-medial progenitors seem to mature differently, emphasizing the different features of the two domains.

- *The different lateral progenitors could reflect the steps necessary for adult neural stem cells formation*

The other major piece of information that these preliminary results highlight is the different progenitor subtypes that are present at the adult lateral VZ and that could represent the different steps necessary to generate mature aNSCs. Indeed, we report here that, both during development and in the adult, the same series of progenitor subtypes – ie. first, non-glial *her4*-negative progenitors, then, non-glial *her4*-positive progenitors, and finally, glial *her4*-positive progenitors – is present at the postero-lateral edge of the pallial VZ (Figure 32).
The transition from NE to RGCs corresponds to what is commonly admitted as how the progenitors change over time during development. However, no clear link has been made so far between *her4*/*Hes5* expression, and the NE/RG transition. Correlations in terms of timing suggests that *Hes5* activation in the mammalian central nervous system is related to the appearance of RG phenotype (Hatakeyama and Kageyama, 2006); but no double labelling of RGCs and *Hes5* expression has been performed in the mouse embryo so far. In the zebrafish, *her4* expression is induced at early stage in progenitors composing the neural plate (Takke et al., 1999), but, as discussed above, when this occurs compared to the NE to RG transition remains unclear in the dorso-medial pallium. In the lateral progenitor population, it seems that only non-glial *her4*-positive and *her4*-negative cells are present at larval stage but the precise timing of the apperearence of glial features in the lateral progenitors would be interesting to address to really understand the delay between the emergence of RGCs and *her4* expression in lateral progenitor maturation.
Interestingly, the NE to RG transition is considered so far as an exclusively embryonic step (Anthony et al., 2004). We report here that, the same series of progenitors in the late juvenile and adult postero-lateral VZ (Figure 32). The lineage tracing experiments we conducted previously already suggested that this pallial region is an adult site of aNSCs formation (see *Dev Cell* publication – section 1.4.7). Altogether, this indicates that in a mature brain, these different progenitor states co-exist and could highlight adult sites of NSCs formation in the zebrafish pallium. Clonal lineage tracing experiment of each progenitor type in the embryo and in the adult would be necessary to clearly demonstrate the hierarchy and the neurogenic activity of all these different cells; in particular to determine what are the potential difference between the NE cells found in the adult and in the embryo. As an example, we do not report any *her6* expression in the NE cells located in the postero-lateral edge of the adult VZ whereas this gene is expressed in the embryonic roof plate (see *Dev Cell* publication – Figure 4B). Moreover, whether such a series exists in other zebrafish adult brain regions and in the mammalian adult brain remain to be determined.

Finally, the acquisition of *her4* expression seems to be correlated with neurogenic activity in the larval and juvenile lateral progenitors. Indeed, *her4* expression appears at 5dpf in the lateral progenitors, a stage before which no lateral neurons are present (see *Dev Cell* publication – Figure 5B-S5C), and no lateral RGCs are reported, indicating that NE cells seems to have a neurogenic activity in the lateral developing domain (Figure 32).

To conclude, the dorso-medial and lateral aNSCs derive from two distincts embryonic neural progenitor populations with different modes of stem cell formation. Even though some steps are similar regarding progenitor maturation such as the acquisition of *her4* expression and the Notch-dependant maintenance, it seems that they do not arise with the same order in the two progenitor populations, with Notch dependency appearing before *her4* expression in the lateral progenitors whereas in the dorso-medial population, *her4* seems to be expressed prior to the emergence of Notch-dependent maintenance. Moreover, the hierarchy of the NE/RG transition, the neurogenic activity, and *her4* expression acquisition seems to be different regarding the pallial VZ region, with neurogenic activity correlated with *her4* expression in the lateral NE cells, whereas it remains to be clarified whether *her4* expression is related to the NE/RG transition and appears before of after the neurogenic activity in the dorso-medial domain.

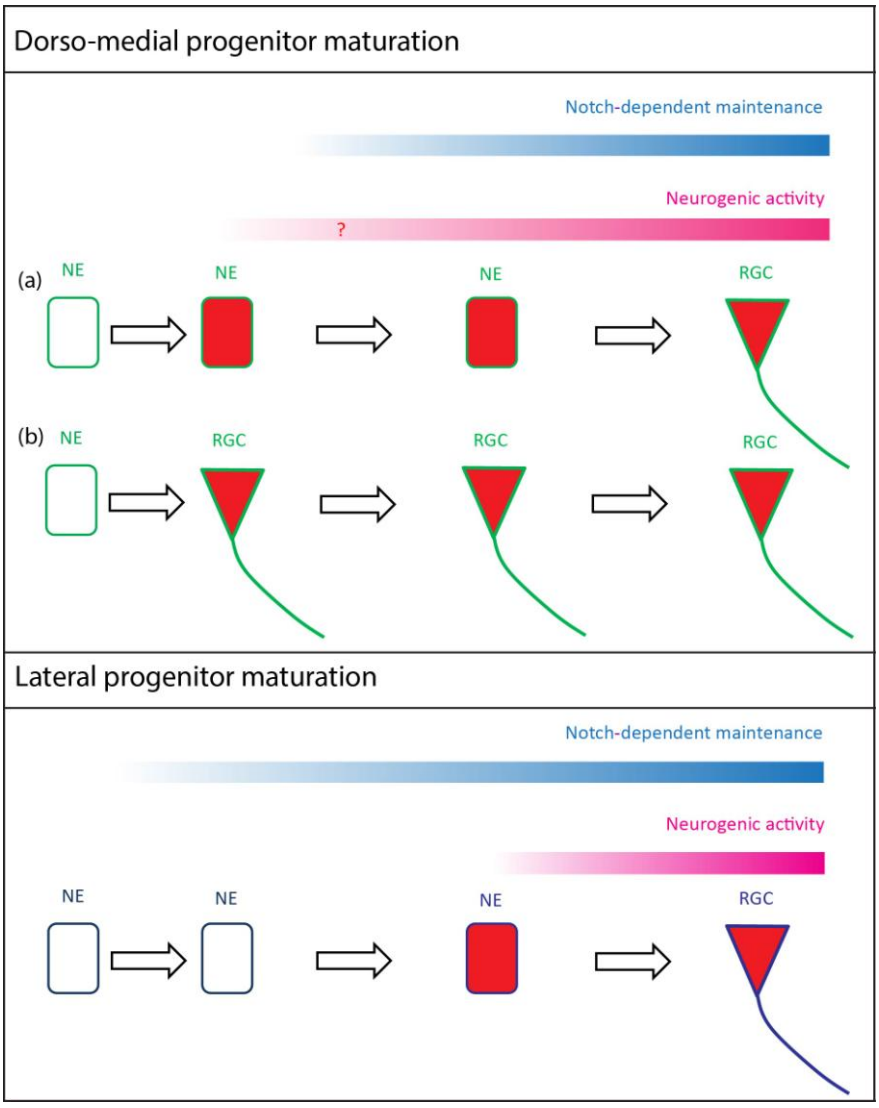


Figure 32: Working model of the neural progenitor maturation of the dorso-medial and lateral VZ

In the dorso-medial VZ, maturation steps occur at early embryonic stages: some NE cells (NE - green) acquire her4 expression (red) but whether these cells remains NE cells ((a) NE –green/red) or become RGCs ((b) RGCs –green/red) remains unclear. Later, they start to be maintained by the Notch pathway, at least for the dorsal progenitors. It is still difficult to determine whether the neurogenic competence arises with the Notch dependency or with her4 expression acquisition in this domain.

In the lateral VZ, the same progenitor series as (a) is visible at juvenile and adult stages but the presence of her4-positive NE cells (NE – dark blue/red) is clear; however, the Notch-dependent maintenance seems to arise before her4 expression (NE – dark blue/red), the latter correlating with the emergence of neurogenic activity.

Finally both lateral and dorso-medial progenitors generate her4-positive, Notch-sensitive radial glial cells (RGC – dark blue/red).

3 Pallium construction

3.1 Introduction

The pallium corresponds to the dorsal part of the telencephalon and hosts the brain regions responsible for higher cognitive functions. In mammals, it contains the isocortex responsible for the integration of environnemental inputs and generation of appropriate behaviors, the hippocampus at the origin of the spatial learning and memory, and the piriform cortex involved in the olfactory system (Kandel et al., 2000).

The isocortex is composed of six layers with an inside-out organization, ie. the youngest neurons are located in the most superficial layers of the cortex. This organization is made possible first, by the scaffold formed by the RGCs. They possess a long radial process and guide neurons during their radial migration toward the pial surface (Kriegstein and Alvarez-Buylla, 2009). Second, the Cajal-retzius cells, the first neuronal population present in the marginal zone of the cortex, send signals to also guide this radial migration (Pierani and Wassef, 2009)(Bielle et al., 2005).,The hippocampus is a small structure that develops medially and caudally to the isocortex from a small cortical region abutting the cortical hem, an organizing center that orchestrates hippocampal formation (Yu et al., 2014). Contrary to the isocortex, it is composed of a single pyramidal cell layer that is submitted to changes during development in order to create different fields, the cornus ammonis (CA) and the dentate gyrus, which hosts adult hippocampal neurogenesis (Zhao et al., 2008).

Unlike in mammals, little information is available on the organization of the pallium in zebrafish. The zebrafish pallium develops following an eversion process leading to the positioning of the VZ, which contains the progenitor cell bodies, at the surface of the pallium (Adolf et al., 2006; Braford, 2009). The ventricle is closed by a stretching sheet of cells, the tela choroida, attached to the lateral pallium (Nieuwenhuys, 2009). The pallium in organized in nuclei (and not morphological layers), a structuration mode often found in non-mammalian brains (Braford, 2009; Medina and Abellán, 2009). Based on morphological features, a regionalization of the zebrafish pallium has been proposed, with the medial pallium (Dm), the dorsal pallium (Dd), the central pallium (Dc), the lateral pallium (Dl) and the posterior pallium (Dp); however, due to the complex morphology of the zebrafish pallium, homologies of these different regions with mouse pallial domains is still unclear (Braford, 2009). Based on functional experiments using brain lesions in the goldfish, it is nevertheless commonly accepted that the lateral pallium hosts the zebrafish hippocampus-like structure (Vargas et al., 2006), and that the medial pallial domain (Dm) is homologous to the amygdala (Portavella et al., 2004). The latter is emphasized by recent experiments performed in the lab

demonstrating the activation of the Dm domain in drug-seeking conditions (von Trotha et al., 2014).

I was interested in investigating how the adult zebrafish pallium is organized and progressively built during development. I already demonstrated that the late formation of the lateral pallial NSCs leads to an heterochrony in the formation of the dorso-medial and lateral pallial areas (*Dev Cell* publication - section1). In addition to informing us on the embryonic origin of aNSCs, the tracing approaches used in this article allowed us to get information on how the zebrafish pallial neuronal compartments are built during development and organized at adult stage. Moreover, to get an overview of the contribution of the different parts of the VZ to the building of the zebrafish pallium, I coupled the re-examination of the *her4*-positive cells lineage tracing experiments with the lineage tracing of a subpopulation of the *her4*-progenitors, highlighted by *CreERT2* expression in the *TP1Glob:CreERT2* fish line. The TP1Glob synthetic promoter is composed of several Notch responsive elements (RPBJ-binding sites) that should activate *CreERT2* expression in the Notch-activated cells (Ninov et al., 2012). Preliminary results in the laboratory indicate that this promoter is not a faithful Notch reporter in the brain (unpublished data); however, it did identify a subset of *her4*-positive progenitors, allowing us to subdivide the population. These approaches together allowed me to get a first overview of the generation time of the different pallial neurons, and how they are spatially distributed within the zebrafish pallium.

3.2 Results

3.2.1 Lineage tracing of *her4*-positive cells at several time points during development reveal how pallial neurons are organized, and the timing of their generation

We previously compared mCherry expression in *her4*^{switch} fish treated with tamoxifen at different stages up to 4.5dpf, and observed that the ventricular mCherry expression boundaries were the same in all these treatments, indicating that there is no *de novo her4* induction in progenitors at the origin of the pallial aNSCs up to 4.5dpf (see *Dev Cell* publication – section 1.4.4). Contrary to the ventricular region, the neuronal mCherry-positive compartment changes depending on the 4-OHT treatment stage. Indeed, in adult *her4*^{switch} fish recombined as embryos between 1ss and 10ss, the large majority of medial pallial neurons express mCherry, but, in contrast, in adult fish recombined as embryos at 1dpf or 2dpf, some pallial neurons (expressing the neuronal marker HuC/D, not shown) located in the most ventral part of the pallium, at the pallial-subpallial boundary, do not express mCherry (Figure 33A). Thus, these neurons would be generated from *her4*-positive

progenitors between 1ss and 10ss. By analyzing the different ventral mCherry pallial boundaries of *her4*^{switch} fish treated at 5dpf and 15dpf, we can observe a dorsal shift of mCherry expression: the later the 4-OHT treatment is performed in *her4*^{switch} fish, the more dorsal the adult mCherry boundary is located in the dorso-medial domain. Analysis of the progeny of *her4*-positive cells at 15dpf indicates that only the most dorsal part of the dorso-medial pallium expresses mCherry in the adult (Figure 33B). This region is thus formed between 15dpf and 3mpf. This highlights that, in the dorso-medial domain, the neurons pile up during development and are thus organized in the adult in sort of “layers” reflecting their timing of generation. Concerning these recombinations at 15 dpf, it is worth noting that although we did not observe any mCherry-positive cell body in the most central part of the pallium, we can clearly see mCherry expression in the parenchyma which corresponds to the neuronal tracts of the mCherry-positive neurons possibly coming from the dorsal pallial neurons (Figure 33B).

Concerning the lateral pallium, it originates from progenitors that start to express *her4* progressively from 5dpf onward, as the mCherry expression is detected in the lateral neuronal compartment of *her4*^{switch} fish only when the treatment is performed after 5dpf (see *Dev Cell* publication – section 1.4.6). We already mentioned that a treatment of *her4*^{switch} fish at 5dpf labels the first third of the lateral pallial ventricular zone (see *Dev Cell* publication – section 1.4.6). Analysis of the lateral neuronal compartement in such fish indicates that a large lateral domain expresses mCherry (Figure 33B – green stars and lines), with a shape similar to the clones described in the experiment using the brainbow system (see *Dev Cell* publication – section 1.4.2); thus, this neuronal population derives from a very restricted number of lateral embryonic progenitors. Interesingly, when we compare the dorso-lateral pallial region in the *her4*$^{switch,\ T(5dpf)}$ and in the *her4*$^{switch,\ T(15dpf)}$, we observe that mCherry expression is similar (Figure 33B – green stars and lines), whereas the most intermediate part of the lateral domain expresses mCherry only after a treatement at 15dpf (Figure 33B – magenta stars and lines). Interestingly, analysis of *her4*^{switch} treated at 1dpf, in order to visualize the dorso-medial/lateral pallial boundary, and treated later at 1.5mpf shows that a thin layer of neurons borders the lateral ventricular zone (Figure 33C), and that some ventro-lateral neurons are for the first time recombined in the pallium (Figure 33C – white circles). Altogether, this indicates that the vast majority of the lateral pallial neurons are formed between 15dpf and 1.5mpf with only the most superficial and ventro-lateral part of the pallium formed after 1.5mpf, and that they seem to be organized in the same way than in the dorso-medial domain, ie. they pile up with the youngest neurons located dorsally.

We can thus conclude, even though the generation of the dorso-medial and lateral pallial neuronal domains is heterochronic (see *Dev Cell* publication – section 1.4.5), that these

domains seem to be organized in the same way with “layers” reflecting the neuronal generation timing.

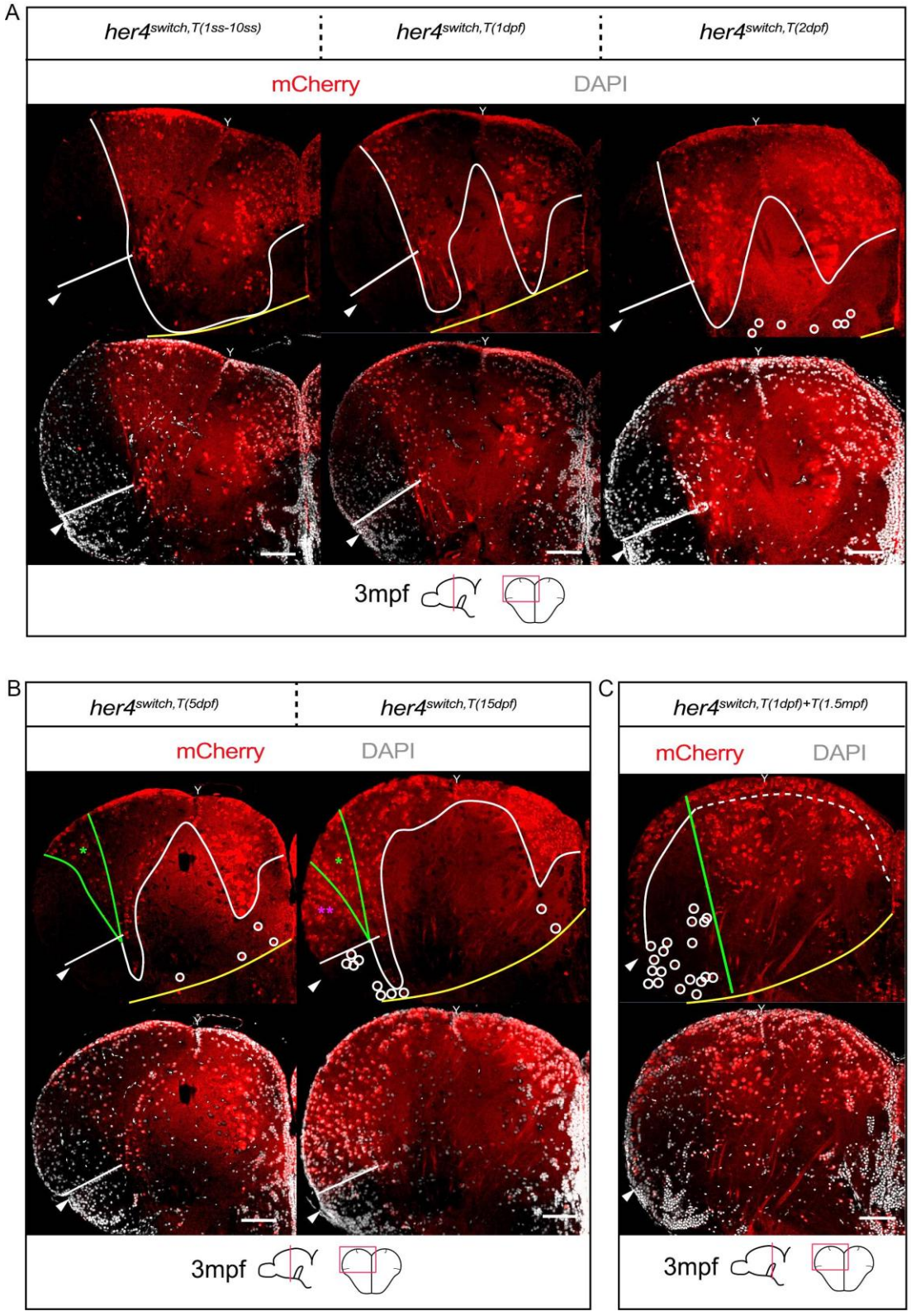


Figure 33: Pallial neurons pile up during development in both the dorso-medial and lateral domains.

(A) Adult fate of progenitors expressing her4 between 1ss and 10ss (her4$^{switch,\ T(1ss\text{-}10ss)}$), 1dpf (her4$^{switchT(1dpf)}$) or 2dpf (her4$^{switch,T(2dpf)}$): respective cross-sections of adult telencephali immunostained as indicated. White lines indicate the mCherry-positive boundary of the neuronal dorso-medial pallial compartment observed after recombination at different stages.

(B) Adult fate of progenitors expressing her4 at 5dpf (her4$^{switch,\ T(5dpf)}$) or 15dpf (her4$^{switchT(15dpf)}$): respective cross-sections of adult telencephali immunostained as indicated. White lines indicate the mCherry-positive boundary of the neuronal dorso-medial pallial compartment observed after recombination at different stages. The green and magenta stars and lines highlight respectively the population of neurons generated from lateral progenitors starting to express her4 at 5dpf and 15dpf.

(C) Adult fate of progenitors expressing her4 at 1dpf and 1.5mpf (her4$^{switch,\ T(1dpf)+\ T(1.5mpf}$): respective cross-section of adult telencephalon immunostained as indicated. The white line indicates the mCherry-positive boundary of the lateral neuronal pallial domain observed after recombination at 1.5mpf, and the dotted white line the hypothetical mCherry-positive boundary of the dorso-medial neuronal pallial domain. The green line highlights the dorso-medial/lateral pallial boundary.

The white arrowhead indicates the end of the glial VZ and the straight white bar the position of the lateral sulcus. Y: sulcus ypsiloniformis. The yellow line indicates the pallial-subpallial boundary. White circles highlight the isolated mCherry-positive neurons.

3.2.2 Lineage tracing experiments of CreERT2-positive cells in the *TP1Glob:CreERT2* fish line at 1dpf reveals a dorso-medial subdivision

Lineage tracing of the *her4*-positive progenitors at different time points allowed us to determine the general contribution of the different neurogenic progenitors to the formation of neuronal pallial compartments during development. However, as *her4*-progenitors contribute from very early stages to the formation of the dorso-medial domain, it does not help us understand the specific contribution of the different parts of the dorso-medial ventricular zone to pallium development. I thus investigated the contribution of a subpopulation of dorso-medial progenitors by taking advantage of the *TP1Glob:CreERT2* line, the promoter of which is composed of several Notch responsive elements (RPBJ-binding sites) (Ninov et al., 2012). Indeed, analysis of the *CreERT2* expression at 1dpf indicates that only a subset of *her4*-positive cells express the *CreERT2* transgene in this line. Specifically, the cell population expressing both *her4* and *CreERT2* is located in the dorso-medial part of the embryonic telencephalon and is not in contact with roof plate cells (Figure 34). We thus investigated the contribution of this *CreERT2*-positive dorso-medial subpopulation to the adult germinal zone.

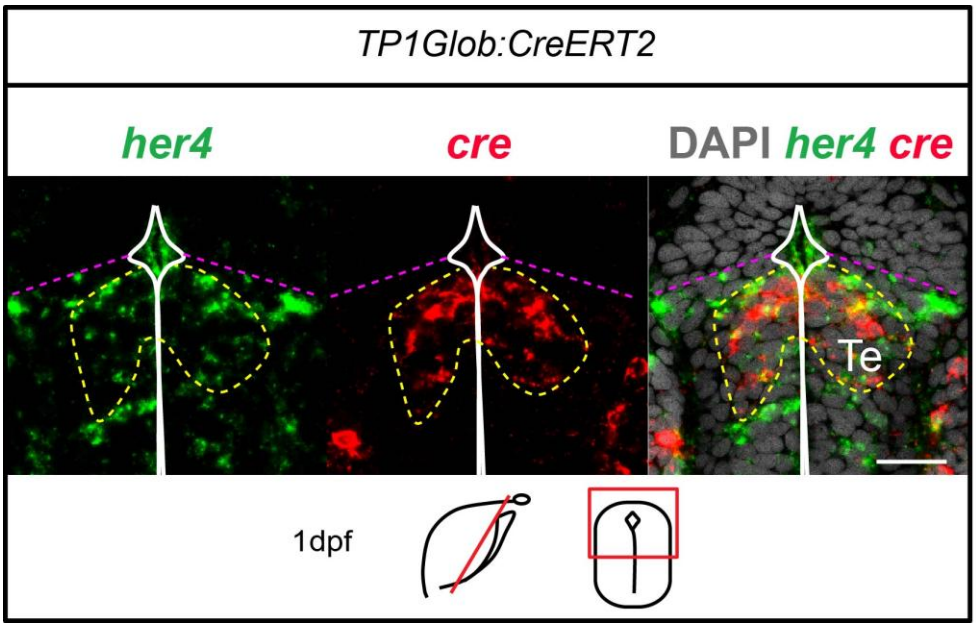
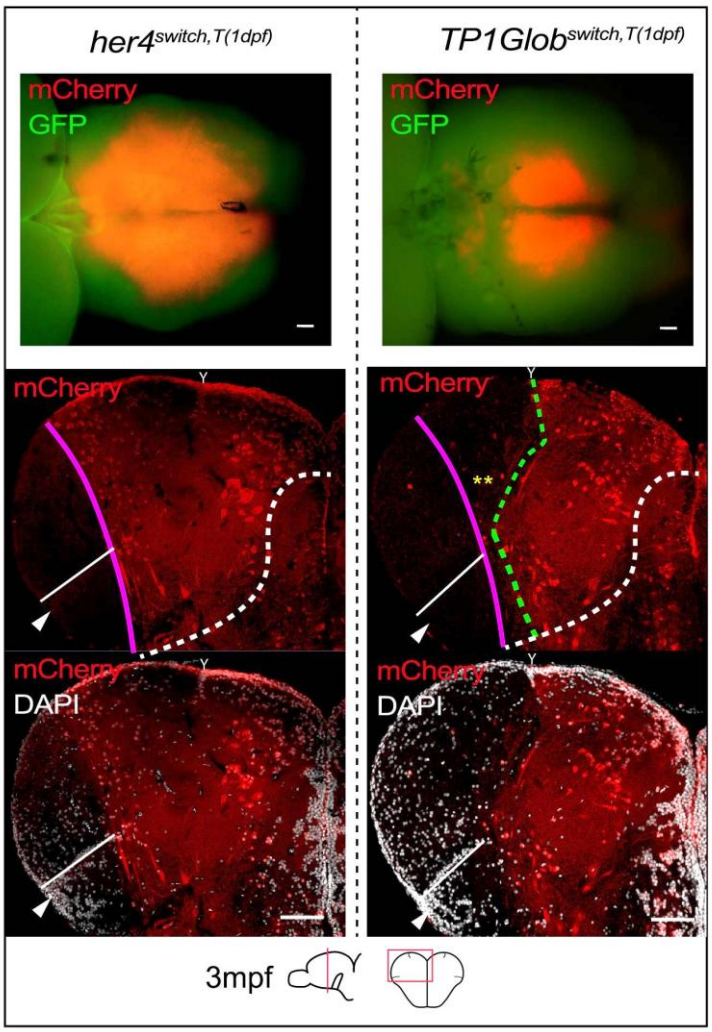


Figure 34: Only a subset of her4-positive cells express CreERT2 in the TP1Glob:CreERT2 line at 1dpf

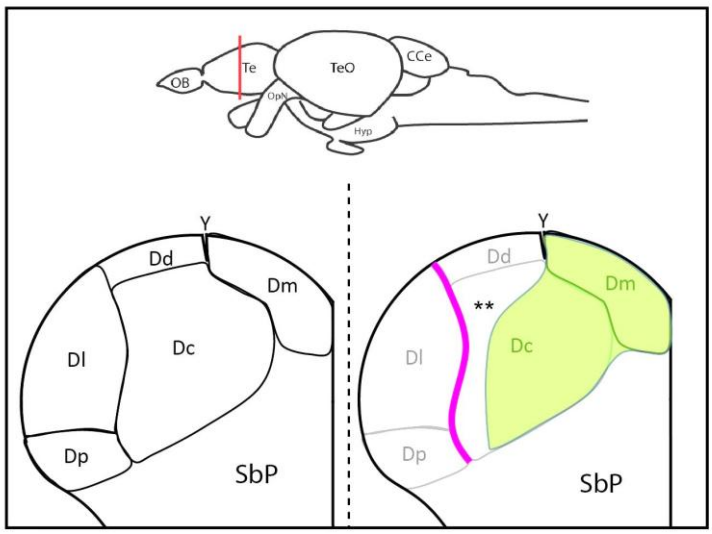
Compared expressions of her4 and creERT2 along the posterior telencephalic ventricle at 1dpf, in a TP1Glob:CreERT2 fish embryo, revealed by fluorescent ISH. Frontal single confocal planes are shown. Yellow dotted lines highlight the CreERT2-positive telencephalic domain and the magenta dotted line indicates the boundary with the roof plate. Plain lines : ventricle; Te: telencephalon.

In order to determine the adult progeny of the early embryonic *CreERT2*-positive cells of the *TP1Glob:CreERT2* fish at 1dpf, I performed a 24-hour 4-OHT treatment on *TP1Glob:CreERT2; ubi:switch* double transgenic fish (hereafter called *TP1Glob*^{switch}) at 1dpf and analysed the pallial mCherry expression at the adult stage (see *Dev Cell* publication for the procedure – section 1.6.2). Contrary to the results obtained with the *her4:ERT2CreERT2* line, the ventricular mCherry expression is restricted to the most medial part of the dorso-medial domain in *TP1Glob*$^{switch,\ T(1dpf)}$ fish (Figure 35 A), indicating that *CreERT*2 is expressed in the progenitors at the origin of the medial population of adult neural stem cells of the dorso-medial pallium. Thus, this experiment allows us to investigate the contribution of the medial ventricular zone to the generation of pallial neuronal compartments. By analyzing mCherry expression in the parenchyma, we observed that it is confined to the most medial and ventral parts of the dorso-medial pallium and thus separates the dorso-medial pallium into two territories (Figure 35 A, green line). It is worth noting that the medial VZ (Dm VZ) does not seem to generate the entire central part of the pallium (Figure 35 A-B, Dc domain). Indeed, we can clearly observe that a small region of the central pallium is mCherry-negative in the *TP1Glob*$^{switch,\ T(1dpf)}$ adult fish (Figure 35 A-B, yellow or black double stars), whereas this region expresses mCherry in the *her4*$^{switch,\ T(1dpf)}$, indicating that this domain likely derives from the dorsal VZ of the dorso-medial domain.

A


her4switch, T(1dpf)
TP1Globswitch, T(1dpf)
mCherry
GFP
mCherry
GFP
mCherry
Y
mCherry
Y
**
mCherry
DAPI
mCherry
DAPI
3mpf

B


Te
TeO
CCe
OB
OpN
Hyp
Y
Dd
Dm
Dl
Dc
Dp
SbP
Y
Dd
Dm
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Dl
Dc
Dp
SbP

Figure 35: TP1-positive cells at 1 dpf generate the medial ventricular zone and most of the central parenchyma

A: Adult fate of progenitors expressing her4 (her4$^{switchT(1dpf)}$) or TP1 (TP1$^{switchT(1dpf)}$) at 1dpf, dorsal view at the top and respective cross-sections of adult telencephali at the bottom, immunostained as indicated. White dotted lines indicate the pallial/subpallial boundary and the green dotted line the dorso-medial/lateral boundary.
The white arrowhead indicates the end of the glial VZ and the straight white bar the position of the lateral sulcus. The yellow double stars indicate the central pallial domain (Dc) that does not derive from the medial ventricular zone (ventricular part of Dm).
B: Right hemisphere of adult zebrafish telencephali at the level indicated in red in the lateral view of the entire adult zebrafish telencephalon, with only the commonly admitted pallial regionalization (left), or with the pallial regionalization combined with the progeny of the TP1-positive cells at 1dpf (TP1$^{switchT(1dpf)}$) highlighted in green. The boundary separating the dorso-medial and lateral domains is highlighted in pink. The black double stars indicate the central pallial domain (Dc) that does not derive from the medial ventricular zone (ventricular part of Dm).
Dp: area dorsalis telencephali pars posterior, Dl: area dorsalis telencephali pars lateralis, Dd: area dorsalis telencephali pars dorsalis, Dm: area dorsalis telencephali pars medialis, Dc: area dorsalis telencephali pars centralis. Y: sulcus ypsiloniformis. SbP: subpallium

Altogether, these results demonstrate that both dorsal and medial parts of the ventricular zone participate in the formation of the central pallium of the zebrafish telencephalon and generate two different juxtaposed territories within the dorso-medial pallial domain.

3.3 Conclusion and preliminary discussion

Analysis of the progeny of progenitors expressing *her4* at different time points allowed us to determine that the pallial aNSCs are generated in different waves from distincts progenitor populations. So far, we focused on the formation of the progenitors but the genetic strategy we adopted allowed us also to get new information on how the pallium is compartmentalized. I used two complementary approaches to investigate the relationship between pallial neurons and the pallial ventricular zone. On the one hand, I reexamined the results from the different lineage tracing experiments of *her4*-positive cells in order to get an overview of when the different pallial neurons were generated during development, and on the other hand, I performed the lineage tracing experiment of a subset of *her4*-positive cells, labeled by *TP1Glob:CreERT2*, in order to determine the relative contribution of the different part of the dorso-medial VZ to the pallial parenchyma.

- *Contribution of the dorsal and medial VZ to parenchymal formation*

By focusing on parenchymal mCherry expression in the different experiments tracing *her4*-positive progenitors, I concluded that, over time, the newborn neurons pile up in the dorso-medial parenchyma. This allows identifying regions within the parenchyma and approximating the stage at which their neurons have been generated, thus highlighting the gradient of maturation of pallial neurons.

First, I determined that the most central part of the pallium corresponds to the oldest pallial neuronal population, mainly formed before 5dpf (Figure 36 – pink, purple). It is interesting to note that even though this region is quite large, it seems to display a low density of neuronal cell bodies and a high quantity of fibers. This is in agreement with the timing of their generation as larvae up to 5dpf have a very small size and thus contain a small number of neurons that are very packed in the parenchyma at that stage. As the brain grows, new neurons generated by the VZ pile up, do not go deep in the parenchyma and develop new brain connections. Thus, a lot of neuronal fibers seem to invade the area and participate in the enlargement of the size of the central pallium.

Second, during juvenile stages up to 15dpf, *her4*-positive progenitors seem to contribute only to the generation of the dorso-medial domain (Figure 36 – pink, purple, blue). This observation could be contradictory with the fact that *her4* is induced in the lateral progenitors from 5dpf onward and that we observed already some lateral neurons at 5dpf and 15dpf (see *Dev Cell* publication – section 1.4.5). However, this corresponds to a very low number of neurons, and the analysis at 3mpf of lineage tracing experiments may not have the resolution required to visualize this population. A second explanation would be that we only performed this analysis at one anterior/posterior level of the telencephalon, and we cannot exclude that these progenitors contribute to the generation of a telencephalic neuronal population located at another antero-posterior level of the pallium or the subpallium. Third, we cannot exclude that these neurons are compacted along the mCherry boundary by a passive or an active mechanism.

The other main finding emerging from these results concerns the contribution of the different parts of the VZ to the formation of the pallial parenchymal domain. Analysis of the adult progeny of *CreERT2*-positive cells in *TP1Glob:CreERT2* fish at 1dpf indicates that the medial VZ mainly contributes to the formation of the central pallium (Figure 36 – white dotted line). Moreover, by making parallels between the mCherry expression profiles of *TP1Glob*$^{switchT(1dpf)}$ and the *her4*^{switch} recombined at different time points, we can conclude that the medial progenitors are the main contributors of embryonic pallial neuronal generation, as the dorsal VZ seems to contribute massively to the pallial neuronal domain only from 5dpf onward (Figure 36 – domain between the white dotted and red lines). This highlights that, in addition to the heterochrony of development present between the lateral and the dorso-medial

domains (see *Dev Cell* publication - section1), there are differences within the dorso-medial domain itself: the medial and the dorsal progenitors do not contribute equally and at the same time to the generation of the dorso-medial neuronal compartment; thus, a heterochrony of development seems to be present also between the dorsal and medial compartments.

- *Contribution of the lateral VZ to parenchymal formation*

In agreement with our conclusions on the formation of lateral aNSCs of the pallium, the lateral neuronal domain is mainly formed between 15dpf and 1.5mpf whereas the majority of the dorso-medial domain is formed up to 15dpf (Figure 36 - green). Even though quantification of the number of neurons formed before and after 15dpf would be necessary to confirm this observation, this re-analysis of neuronal compartment formation emphasizes the heterochrony that exists in the formation of the two pallial neuronal domains.

After 1.5mpf, the generated neurons compose the most superficial pallial neuronal layer of the lateral domain, as well as most of the ventro-lateral portion, corresponding to the latest pallial domain to be formed. Moreover, like for the dorso-medial domain, very little neuronal migration takes place in the lateral pallial parenchyma. This idea was already suggested from BrdU pulse/chase experiments at adult stage which showed that newly born BrdU-positive neurons acquire functional markers such as *gad67* close to the ventricular zone (Adolf et al., 2006), and this situation is similar in reptiles, with a short migration of adult-born neurons through the parenchyma (Pérez-Cañellas and García-Verdugo, 1996).

The present study allows us to build a working model on the contribution of the different parts of the VZ to the development of neuronal compartments up to the adult stage. We report here that, similarly to the late juvenile- and adult-born neurons, the embryonic and larval neurons seem to stay close to their site of generation. Interestingly, comparison between the organization of the zebrafish pallial neuronal compartment and the mouse cortex highlights that, despite the different mode of neuronal generation with the everted VZ and the restricted neuronal migration, the general organization of both zebrafish and mouse neuronal domains is similar with the oldest neurons located in the inner layer and the youngest neurons being in the most supercifial position. Whether this has a functional significance remains to be determined.

Moreover, it is important to keep in mind that the pallium, in addition to its planar organization, is also compartimentalized along the anterior/posterior axis and we already demonstrated that the lateral pallium derives from a very restricted number of progenitors that massively amplify and sequentially generate neurons with an antero-posterior orientation. This organization highlights that a gradient of antero-posterior growth also exists in the pallium. This working model can, at least, be reasonnably applied to the anterior pallium as the mCherry expression pattern of the different *her4*-positive lineage tracing

experiments is quite similar between the anterior and the medial pallium (not shown). We have shown that the postero-lateral pallium continues to generate aNSCs *de novo* in the adult (see *Dev Cell* publication - section 1.4.7) and thus would contain a higher proportion of "young" newborn neurons than the rest of the pallium. Nevertheless, lineage tracing experiments of progenitors differentially positioned along the A/P axis would be necessary to really get an overview of the timing of neuronal generation in the anterior *versus* posterior pallium.

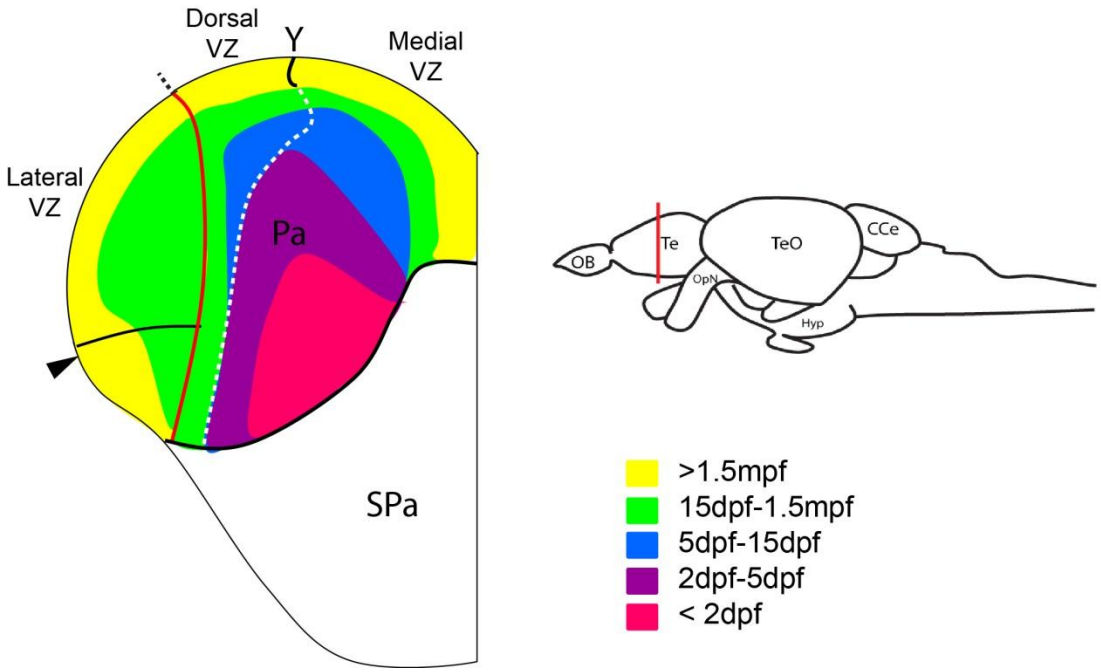

Figure 36 : Working model of the timing and spatial organization of pallial neuronal generation during development up to 1.5mpf

Right hemisphere of an adult zebrafish telencephalon at the level indicated in red in the lateral view of the entire adult zebrafish brain, with the different neuronal generation timings emerging from the comparison of mCherry expression in the different lineage tracing experiments of her4-positive cells (correspondence between stages and colors as indicated). The mCherry parenchymal boundary of the TP1$^{switchT(1dpf)}$ fish is highlighted by the white dotted line. The red line and the small black dotted line correspond to the dorso-medial/lateral pallial boundary respectively in the parenchyma and the ventricular zone. The black arrowhead indicates the lateral edge of the VZ. The black line highlights the position of the lateral sulcus. Pa:Pallium; SPa: Subpallium;Y: sulcus ypsiloniformis

CHAPTER III: DISCUSSION

CHAPTER III: DISCUSSION

The work presented in this thesis aimed to identify the embryonic neural progenitor populations at the origin of the aNSCs of the zebrafish pallium. The first part of my PhD project was dedicated to performing the lineage tracing analysis of the actively neurogenic progenitors, characterized by *her4*-expression, using genetic strategies and analyzing their entire pallial progeny during development and at adult stage. The results obtained highlight: (i) the embryonic origin of the dorso-medial aNSCs, which derive from progenitors that continuously express *her4* during development up to adult stage, (ii) the progressive *her4* activation from post-embryonic stages onward in progenitors at the origin of the lateral aNSCs. In a second step, I further characterized the embryonic progenitor population at the origin of the lateral aNSCs by combining clonal analyses with spatially restricted recombinations. The results obtained revealed that the lateral VZ of the adult pallium derives from NE progenitors expressing other *her* genes, such as *her6* and *her9*, namely from « progenitor pools » progenitors located in the embryonic telencephalic roof plate. By performing pharmacological treatments with a γ-secretase inhibitor, I could test the role of the Notch pathway in regulating the maintenance of the lateral and dorso-medial neural progenitors along development. These results highlight that, while dorso-medial progenitors are maintained by Notch signaling from embryonic stages onward, lateral progenitors are first maintained independently of the Notch pathway and then generate progenitors that display a Notch-dependent maintenance.

By combining these results with the timing of *her4* expression in the dorso-medial and lateral progenitors and the analysis of the progenitor features such as their NE/RG nature and their neurogenic activity, we were able to compare the maturation steps of the two embryonic progenitor populations, which end up forming similar aNSCs.

Finally, in addition to stem cells origin, these data provided general information on how the zebrafish pallium is built, with the position of adult-born neurons reflecting their birth date, a heterochony in the development of the medial, dorsal and lateral pallium. These data further provide information on homologies between zebrafish and mouse pallial areas, and particularly emphasize the homology of the lateral zebrafish pallium with the mammalian hippocampus.

1 Origin of adult pallial neural stem cells

The lineage tracing approach we used for this work allowed us to determine that the embryonic origin and mode of formation of aNSCs diverge depending on the pallial region. First, pallial aNSCs derive from different embryonic neural progenitor populations: dorso-medial aNSCs originate from early neurogenic progenitors located along the pallial posterior wall, while lateral aNSCs emerge from non-neurogenic embryonic progenitors located along the telencephalic roof plate. Second, this differential embryonic origin imprints differences regarding timing of progenitor activation and mode of stem cells generation. We will discuss below first the link between embryonic regionalization and adult pallial progenitor generation, in relation with the different lineage tracing experiments already done in the mouse system, and highlight the common features regarding aNSCs in the zebrafish and mouse adult neurogenic zones. Then, I will integrate the different results concerning the role of the Notch pathway and *her4* expression in progenitor maintenance and neurogenic activity in the both dorso-medial and lateral VZ, and compare it with the key role of this pathway in mouse progenitors. And finally, I will discuss the different modes of pallial NSCs generation in the zebrafish, and analyze whether “adult sites of NSCs formation”, such as I revealed in the lateral pallium, could exist in other zebrafish brain regions, and in other vertebrates.

1.1 Spatial contribution of embryonic neural progenitors to adult pallial neural stem cells heterogeneity

1.1.1 The aNSCs of the dorso-medial pallium derive from *her4*-positive embryonic progenitors.

Our genetic approach to lineage trace pallial *her4*-positive progenitors allowed us to precisely determine the subpopulation of embryonic progenitors at the origin of the pallial NSCs located in the adult dorso-medial domain. After a precise comparison of the endogenous *her4* expression with *ERT2CreERT2* expression in the *her4:ERT2CreERT2* fish line and with some regional markers such as the subpallial marker *gsh2* or the pallial neuronal marker *tbr1*, we were able to localize embryonic *her4*-positive pallial progenitors at the origin of the aNSCs of the dorso-medial pallium to the dorsal part of the posterior wall of the telencephalic ventricular zone (Figure 37A). In the mouse, lineage tracing of the pallial embryonic progenitors located at E10 within the *Emx1*-positive domain indicates that they contribute to the formation of the pallial aNSCs, as they participate to the adult dorsal SEZ formation (Willaime-Morawek et al., 2006; Young et al., 2007). In the mouse embryo, *Emx1* is expressed from E12.5 until birth in pallial progenitors except in the ventral pallium (Medina et al., 2004), and in the zebrafish, *emx1* is also expressed in the embryonic telencephalon

and its activation starts at very early stage, 15-20ss (Kawahara and Dawid, 2002); it could be interesting to compare ventricular *emx1* and *her4* expression in the embryonic zebrafish telencephalon to determine whether *emx1* expression characterizes a subpopulation among *her4*-positive progenitors that could correspond to the progenitors at the origin of the region homologous to the dorsal SEZ.

The lineage tracing experiment that we performed using the *TP1Glob:CreERT2* fish line allowed us to subdivide the *her4*-positive embryonic VZ into two progenitor populations: the progenitors that generate the medial aNSCs, and the progenitors, located between the medial population and the roof plate, which generate the dorsal aNSCs. However, it is still unclear to which mouse pallial embryonic regions these embryonic progenitors are homologous to, and comparison of *emx1* with *CreERT2* expression in the *TP1Glob:CreERT2* line could also help us refine the embryonic ventricular regionalization and progenitor population at the origin of aNSCs.

1.1.2 Further parallels between the embryonic origin of the mouse SEZ and the zebrafish adult VZ

In addition to *Emx1* lineage tracing, the contribution of *Dbx1*-positive, *Gsh2*-positive and *Nkx2.1*-positive progenitors to the adult mouse SEZ has been reported (Young et al., 2007). *Dbx1* is expressed in the mouse embryonic telencephalon at the pallial-subpallial boundary and in the septum, and the *Dbx1*-positive cell population contributes to the regionalization of the embryonic cortical area via the production of Cajal-retzius cells (Griveau et al., 2010). In the zebrafish, both *dbx1a* and *dbx1b* are not expressed in the embryonic telencephalon but it is expressed in the intermediate compartment of the spinal cord (Gribble et al., 2007), suggesting that in the zebrafish spinal cord as well, it is important for the development of the intermediate compartment. It is worth noting that the lineage tracing experiment performed in the mouse telencephalon is not based on an inducible genetic strategy, and thus does not allow determining precisely whether an early or late Dbx1-positive population is at the origin of the dorso-lateral SEZ. We cannot exclude that *dbx1* would be expressed later in progenitors at the origin of some part of the adult telencephalic VZ in the zebrafish. Establishing precise expression data and then lineage tracing experiments would be necessary to investigate this question.

The *Gsh2* and *Nkx2.1* subpallial markers are expressed in progenitors generating the lateral and ventral SEZ (Young et al., 2007). Our analysis of *her4* expression indicates that it is expressed in the embryonic subpallial progenitors of the zebrafish telencephalon; however, the *her4* promoter fragment used to generate the *her4*:*ERT2CreERT2* line does not drive a Cre expression similar to the endogenous *her4* expression pattern in the subpallial

compartment, preventing us from analyzing the contribution of the subpallial embryonic progenitors to the adult telencephalic ventricular zone.

The mouse SEZ generate neuroblasts that migrate towards the olfactory bulb to generate olfactory interneurons (Kriegstein and Alvarez-Buylla, 2009). Lineage tracing of Gsh2- and Emx1-positive cells indicated that both contribute to the generation of progenitor populations that produce the rostral-migratory stream (RMS) neuroblatsts (Young et al., 2007), highlighting that both pallial and subpallial progenitors contribute to the RMS. Interestingly, in the zebrafish, a RMS-like stripe that reach the olfactory bulb has been described with non-glial/PSA-NCAM-positive dividing cells (März et al., 2010b). However, our lineage tracing experiments of *her4*-positive cells during development have never indicated a contribution of the *her4*-positive population to the RMS-like stripe cells. Moreover, in the adult telencephalon, these cells express *her9* (Chapouton et al., 2011), indicating that possibly, neuroblasts of the RMS-like stripe in the zebrafish could derive from embryonic *her9*-positive progenitors without going through a *her4*-positive cell state.

In addition to regional markers in the mouse, lineage tracing experiments indicate that Shh-responding cells (*Gli1*-positive cells) at E15 (Ahn and Joyner, 2005), as well as cells responding to Wnt signaling (*Axin2*-positive cells) at E12.5 (Bowman et al., 2013), contribute to the emergence of the adult SEZ in mouse. The role of Wnt signaling in the development of the mouse pallium has been reported as it participates in the patterning of the embryonic pallium but also promotes the proliferation of cortical progenitors during development (Bielen and Houart, 2014). Similarly, in the zebrafish, *wnt8b* has been shown to promote formation of the pallial compartment at the expense of subpallium in the telencephalon by participating in the dorsal inhibition of *foxg1* expression at early stages of development (Danesin et al., 2009). Thus, potentially, some Wnt-responding cells could participate in the formation of the dorso-medial VZ in the zebrafish pallium as well. Moreover, in agreement with mouse lineage tracing results, analysis of the expression profiles of *axin2,* as well as *gli1,* indicate that they are expressed in the embryonic telencephalon (Thisse et al., 2001), but precise analyses would be necessary to confirm that they are at least partially co-expressed with *her4* transcripts in the zebrafish telencephalon. In addition, concerning the origin of the zebrafish RMS-like stripe, expression pattern of *gli1* combined with lineage tracing of *gli1*-positive cells could also indicate the population homologous to the one in the mouse that contributes to the generation of RMS neuroblasts.

1.1.3 The lateral pallial adult neural stem cells derive from telencephalic roof plate cells

Contrary to the dorso-medial VZ, the aNSCs of the lateral pallium do not derive from *her4*-positive embryonic neural progenitors. Long-term clonal analyses combined with spatially restricted lineage tracing of roof plate cells indicate that the adult lateral pallial VZ derives from a restricted number of progenitors located in the roof plate at 1dpf (Figure 37A). This *her4*-negative region expresses several Wnt ligands such as Wnt8b or Wnt3a indicating that these factors could play a role in the emergence of lateral aNSC. Interestingly, in the mouse, Wnt ligands are key components of the cortical hem, a signaling center essential for hippocampal development as it allows proliferation of the caudo-medial cortex from which the hippocampus emerges (Lee et al., 2000). Lineage tracing of Wnt-responding cells at E12.5 in the mouse highlights that these cells contribute to generate most of the adult SGZ (Bowman et al., 2013), and *Wnt3a* expression has also been shown in adult progenitors of the mouse SGZ, and is a key regulator of adult hippocampal neurogenesis (Lie et al., 2005). Similarly, we report that, in the zebrafish, *wnt3a* is expressed at the postero-lateral edge of the pallial VZ at late juvenile (1.5mpf) and adult (3mpf) stages.

Wnt3a expression in the hem results from BMP signalling present in the adjacent choroid plexus (Shimogori et al., 2004b). Interestingly, we report that, in addition to *wnt3a*, *bmp6* is expressed in the zebrafish telencephalic roof plate at 1dpf indicating that this region could correspond to the zebrafish “cortical-hem” like structure. In addition to Wnt and BMP signaling, the FGF pathway has been reported to regulate the hem structure, as anterior Fgf8 is responsible for restricting *Wnt3a* expression to the hem (Shimogori et al., 2004b). In the zebrafish, we report that *fgf8* is expressed in the roof plate as well but it could be interesting to determine the exact expression pattern of *wnt3a*/*wnt8b* compared to *bmp6* and *fgf8* to determine whether they are co-localized or expressed in particular subregions of the zebrafish telencephalic roof plate. This could suggest similar roles than in the mouse hem. Preliminary results obtained in the lab would tend to indicate that, as in the mouse, late *bmp6* expression is restricted to the choroid plexus (S.Galant – unpublished results), suggesting a conserved role of BMP signaling in choroid plexus specification. Whether *fgf8* is still expressed later in lateral pallial progenitors remains to be determined.

Finally, it has been proposed that Shh is involved late in SGZ formation. Indeed, *Gli1*-positive cells at late developmental stages (E17.5) contribute to the generation of the SGZ (Ahn and Joyner, 2005), and derive from a population located first in the ventral hippocampus that later migrate dorsally to generate the dentate gyrus (Li et al., 2013). Moreover, the Shh pathway has been shown to maintain stemness of the post-natal aNSCs (Li et al., 2013; Machold et al., 2003). In the zebrafish, late *gli1* expression has not been thoroughly investigated; however, preliminary results in the lab would suggest that it is expressed in the postero-

lateral edge of the adult pallium (M.Coolen, unpublished results). It would be interesting to characterize the *gli1* expression pattern in the zebrafish lateral pallial VZ up to late juvenile stages, as well as to inhibit the Shh pathway by performing cyclopamine treatments on late juvenile fish, to determine whether Shh implication in SGZ formation is conserved in the development of the lateral pallial VZ.

1.2 Progenitor maturation at the origin of adult neural stem cells

1.2.1 Hes/*her* genes, Notch and pallial progenitor sequence

Our experiments on the lineage of *her4*-positive cells throughout development, combined with investigations on the role of the Notch pathway on neural progenitor maintenance, allowed us to discriminate several steps in progenitor maturation during development, and highlight differences in the timing of the acquisition of NSCs features in the dorso-medial versus lateral pallial VZ.

1.2.1.1 Maturation of dorso-medial pallial progenitors

The dorso-medial aNSCs derive from neural progenitors expressing *her4* from very early stage of development. Indeed, at 10ss already, the *her4*-positive embryonic progenitors comprise cells at the origin of dorso-medial aNSCs, highlighting that already at that stage, the entire population that will generate aNSCs in this region is already expressing *her4*.

However, whether these progenitors derive from cells expressing non-canonical *her* genes earlier than 10ss remains to be determined. As an indication, *her6* seems to be broadly expressed in the forebrain region at 5ss and the earliest lineage tracing experiment of *her4*-positive cells that we performed was at 10ss, suggesting that *her6* progenitors could generate *her4*-positive telencephalic proneural cluster cells in the dorso-medial territory. In the mouse, *Hes1*, homologous to zebrafish *her6*, and *Hes3*, homologous to *her3*, are expressed in the neural plate before *Hes5*, homologous to *her4*, and before the onset of neurogenesis (Hatakeyama and Kageyama, 2006). This indicates similarities in the steps of embryonic neural progenitor maturation between mouse and zebrafish embryos. However, lineage tracing of Hes1/*her6*-positive cells would be necessary to confirm that the first maturation step of dorso-medial pallial progenitor corresponds to a transition from a *Hes1*/*her6*-positive to *Hes5*/*her4*-positive progenitor.

We mention above that even though *Hes5* is activated in the mouse cortical progenitors, *Hes1* is still expressed in this population and is dependent on Notch signaling. It is interesting to mention that *her6* is induced at 5dpf in the dorso-medial progenitors (S.Galant – unpublished results). Interestingly, from this stage onward, the proportion of ventricular

proliferating cells gradually decreases in this area, and it could be interesting to determine whether *her6* could play a role in this increased cell cycle length and in the emergence of quiescent cells in the dorso-medial domain, and whether this potentially *her6*-related process is dependent on Notch signaling.

1.2.1.2 Maturation of lateral pallial progenitors

Lateral neural progenitors derive from cells that do not express *her4* at very early stage of development but that express the non-canonical *her* genes *her6* and *her9*. These progenitors correspond to progenitor pools composed of NE cells that later during development generate *her4*-positive NE and RGCs, the latter corresponding to lateral aNSCs of the zebrafish pallium (Chapouton et al., 2010; März et al., 2010b). Interestingly, it emphasizes the capacity of the progenitor pools to generate, over time, cells with “proneural clusters”-like features, expressing *her4* and producing neurons as already proposed (Geling et al., 2003, 2004).

In the lateral pallial VZ, Notch-dependent maintenance seems to be acquired independently of *her4* expression, at least in some neural progenitors. First, during early development, the entire lateral progenitor population is neither *her4*-positive nor Notch-sensitive but expresses *her6* and *her9* genes, probably involved in maintaining the population. However, the upstream signals controlling their expression remain to be determined (Figure 37B). We already mentioned that Wnt, BMP and FGF pathways are present in the roof plate and whether one of them is involved in maintaining the lateral progenitors would be interesting to address. Preliminary results tend to indicate that FGF would not play a role in this process (H. Oubert – unpublished results).

Second, upon juvenile Notch inhibition, we observe a depletion of both *her4*-positive and *her4*-negative lateral progenitors. This highlights that some lateral progenitors become Notch-sensitive even though they do not express *her4*. It would be interesting to determine what could be the factors involved in maintaining these *her4*-negative/Notch-sensitive progenitors. For this, we could analyze the expression pattern of other canonical *her* genes such as *her2*, *her12* or *her15* among the *her4*-negative/Notch-sensitive late lateral progenitor population.

Finally, in the embryonic roof plate both *her6* and *her9* are expressed and later, only *her9* stays in the NE lateral progenitors. Inhibition of *her6 in* the thalamus via morpholino injections (Scholpp et al., 2009), as well as inhibition of *her9* in the otic vesicle (Radosevic et al., 2011) lead to the activation of proneural genes. Interestingly, experiments at the midbrain-hindbrain boundary have shown that Notch overactivation leads to inhibition of *her5*, another non-canonical *her* gene, and to the upregulation of neurogenic genes (Geling et al., 2004). The possible crosstalk between canonical and non-canonical *her* genes remain to be clarified and in particular whether they inhibit each other. One working model would be

that, in the lateral progenitors, emergence of Notch dependence by intrinsic or extrinsic signals could inhibit non canonical *her* genes and activate canonical *her* genes expression in this population. The latter would reinforce non-canonical *her* genes inhibition, triggering the transition between “progenitor pool” cells to “proneural cluster”-like progenitors, more prompt to generate neurons (Figure 37B).

1.2.2 Neurogenic activity in pallial neural progenitors

1.2.2.1 Intrinsic factors involved in the neurogenic activity and RGCs emergence in the lateral pallial progenitors

- *A potential factor at the origin of the neurogenic switch in the lateral pallial progenitors of the zebrafish: Dmrt2?*

In the mouse, the emergence of Notch signaling in embryonic neural progenitors has been proposed to be related to neurogenic capacity, as *Hes5* expression is found only in neurogenic regions. This also correlates with the appearance of oscillations of *Hes* genes and proneural genes in telencephalic progenitors, under Notch control. However, no direct functional data are available regarding the respective role of Hes5/*her4* and Notch in the acquisition of neurogenic potential (Hatakeyama et al., 2004).

Interestingly, we observed in the zebrafish embryonic lateral pallium a correlation between the acquisition of *her4*-expression and neurogenic capacity. A recent study has shown the implication of one transcription factor in the zebrafish embryo in promoting neurogenesis in the postero-dorsal telencephalon at an early stage of development, the Doublesex- and Mab-3-related transcription factor 2, Dmrt2. These factors comprise a Zinc finger-like DNA-binding motif also called DM domain (Zhu et al., 2000), are important for sexual development in both vertebrates and invertebrates, but are also expressed in the central nervous system (Hong et al., 2007). In the zebrafish, *dmrt2*$^{-/-}$ mutants embryos display an increased *her6* expression and reduced neurogenesis in the dorsal telencephalon (Yoshizawa et al., 2011). It has been proposed that this factor is involved in inhibiting *her6* and promoting neurogenesis by activating *emx3* in the dorsal telencephalon, a gene already demonstrated as necessary for dorsal telencephalic neurogenesis (Viktorin et al., 2009; Yoshizawa et al., 2011). In the mouse, Dmrt proteins have been implicated in the differentiation of the dopamine neurons in the midbrain (Gennet et al., 2011) but no particular role in promoting telencephalic neurogenesis has been reported. It would be interesting to investigate the expression pattern of *dmrt2a* in the pallium before and after 5dpf to see whether it could correspond to a good candidate for the lateral neurogenic switch, as it could repress *her6/her9* and activate *her4*

expression in the lateral pallial progenitor pool, possibly via an action on Notch signaling (Figure 37B).

Moreover, the relation between *her4* expression and neurogenic activity remains to be clarified. Whether *her4* confers only a neurogenic competency or whether all the *her4*-positive progenitors are actively engaged into neuronal generation remains to be addressed. Likewise, it would be interesting to look whether *her4* expression oscillates, as has been shown for *Hes5* and *Hes1* in correlation with neurogenesis competence in neurogenic progenitors.

Live imaging experiments of single progenitor combined with *in vitro* assay would be necessary to address this issue.

- *Link between RGCs emergence and type of divisions in the lateral progenitors*

Once neurogenic activity is present within a progenitor population, different types of cell divisions could co-exist within the progenitors population : symmetric neurogenic, symmetric non-neurogenic, and asymmetric self-renewing. In the mouse, it is though that NE cells perform mainly amplifying symmetric divisions and that RGCs perform asymmetric self-renewing divisions, as it occurs in the cortical progenitors, the emergence of which would be linked with the neurogenic activity in the progenitors (Dimou and Götz, 2014). In the zebrafish embryonic hindbrain at 30hpf, it has been shown that NE cells mainly perform symmetric neurogenic divisions (Lyons et al., 2003). The pallial lateral progenitor population in the zebrafish is composed of neuropithelial cells that, from 5dpf onward, start to produce the first lateral neurons, and later, generate at least some neurogenic RGCs. Whether differences exist regarding the types of neurogenic cell divisions between the NE and RGCs in the lateral progenitor populations during development remain to be determined. Single cell tracing experiments would be necessary to determine the types of cell divisions occurring in the lateral pallial population; we could thus investigate it by performing clones in the population and short-term lineage tracing of their progeny.

In the mouse, several factors are involved in regulating the transition between NE and RGCs and thus are linked with the symmetric/asymmetric cell division transition as well. The transcription factors Sox1, Emx2 and Pax6 are involved in orchestrating this change. Indeed, Sox1 promotes a symmetric and neuroepithelial phenotype, whereas Pax6 is involved in inhibiting Sox1 and triggers the emergence of RGCs and asymmetric cells divisions in cortical progenitors (Suter et al., 2009). Interestingly, *in vitro* experiments indicate that the overexpression of Sox1 inhibits *Hes1* (orthologous to *her6* in the zebrafish) promoter activity, even in the presence of Notch signaling, and co-immunoprecipitation experiments from mouse E10.5 embryo confirm that Sox1 binds the *Hes1* promoter *in vivo* (Kan et al., 2004). This relationship between Sox1 and Hes1/*her6* could be interesting to address in link with the

transition between lateral NE and RGCs, and the potential switch from symmetric to asymmetric divisions (Figure 37B). Indeed, *sox1* could be involved in inhibiting non-canonical *her* genes in the lateral progenitors such as *her6* or *her9* on one hand, but would participate in promoting symmetric divisions in this population on the other hand. Moreover, these authors showed that Sox1 inhibits the β-catenin-mediated TCF/LEF signaling, components of the Wnt signaling pathway, and promotes the expression of proneural genes such as *Ngn1* (Kan et al., 2004), indicating that it could also promote neurogenesis in the lateral progenitors. Our observations that Wnt ligands are expressed in the *her4*-negative roof plate cells and later in the *her4*-negative NE cells maintained in the lateral pallium, whereas both *her4*-positive lateral NE and RGCs do not express Wnt ligands, is compatible with a similar interaction between Sox1 and Wnt to promote neurogenesis in the zebrafish lateral pallial progenitors. The expression pattern of Wnt signaling components would need to be investigated, to identify the Wnt-responding cells in the zebrafish lateral progenitors (Thisse et al., 2001). Similarly, Pax6 and Emx2 repress each other and Emx2 acts in favor of symmetric divisions in the cortical progenitors (Heins et al., 2001) (Figure 37B). Interestingly, Pax6 is expressed in a gradient close to the pallial-subpallial boundary (PSB) and *Emx2* is expressed close to the cortical hem in the mouse, indicating a regional influence of these factors on progenitors. In the zebrafish, *pax6a* (Wullimann and Rink, 2001) and *emx2* (Kawahara and Dawid, 2002) are expressed in the embryonic telencephalon, with *emx2* expressed in the dorsal telencephalon and *pax6a* a at the PSB, but *pax6a* a seems to be restricted to neuronal populations. However, their functions in the zebrafish brain remain to be determined and it would be worth investigating whether later, during juvenile development, they are expressed in the discrete area of the postero-lateral pallium where NSCs are formed and influence the NE to RG transition in the lateral neural progenitors. For instance, we could overexpress *emx2* under the control of a lateral progenitor specific promoter and appreciate whether it increases the number of neuroepithelial progenitors at the expense of the emergence of the RGCs in the lateral population.

- *Asymmetric divisions in the lateral pallium?*

Finally, at least some lateral progenitors could perform asymmetric cell divisions during development as they do in the mouse. To address this question, short-term single cell lineage tracing would first need to be performed. In mouse, asymmetric divisions go through either unequal asymmetric cell fate determinants inheritance in the two daughter cells, or an intra-lineage decision taking place after cell division. *Par3* has already been shown to influence cell fate decision in the zebrafish forebrain at early stage of development by sequestering the Notch-promoting factor Mindbomb in the apical inherited daughter cell that will differentiate into a neuron, allowing the maintenance of a high Notch level into the

remaining progenitor (Dong et al., 2012a). Moreover, Par proteins have been shown to participate in mitotic spindle orientation via their interaction with Insc factor in Drosophila but also in the mouse (Lancaster and Knoblich, 2012). Indeed, mInsc asymmetrically orients the mitotic spindle and its expression has been related to the emergence of RGCs and asymmetric cell divisions in the mouse cortex (Postiglione et al., 2011; Zigman et al., 2005). Together, if we reveal asymmetric divisions taking place in lateral pallial progenitors, then it would be interesting to investigate whether the same Par3/Insc machinery participates in this process.

1.2.2.2 Neurogenic switch in the lateral pallial VZ: a potential role for fish metamorphosis?

Many changes appear at larval stages in lateral pallial progenitors: they start to express *her4*, become neurogenic and start to generate RG cells. All these events could be linked to each other as we discussed above but the remaining question is whether an external signal, such as a systemic cue (and which one), could trigger all these events.

In Drosophila, during the transition from larva to pupa, Ecdysone, a steroid hormone responsible for metamorphosis, is thought to be responsible for switching between a phase of neuroepithelial expansion, mainly controlled by diet, to a neurogenic phase by the emergence of neuroblasts. This occurs via a downregulation of Delta expression in the neuroepithelial population (Lanet et al., 2013), indicating that systemic signals are involved in regulating neurogenic activity in the developing nervous system.

In at least some vertebrates such as the frog, metamorphosis has also been reported and is responsible for the transition between the larval to the juvenile stage (Laudet, 2011). It is triggered by the action of thyroid hormones (Morvan-Dubois et al., 2008). Thyroid hormones (THs) primarily act through thyroid hormone receptors (TRs); TRs bind to the DNA regulatory regions of target genes to activate or repress transcription through interactions with accessory proteins known as co-regulators. Two major THs exist: T3 (3,5,3′-triiodo-l-thyronine) and T4 (3,5,3′,5′-tetraiodo-l-thyronine, also known as thyroxine), and T3 has been assumed to be the active form of TH as it binds the TR with a greater affinity than T4 (Schroeder and Privalsky, 2014). T4 is thus a pro-hormone that circulates in the body to be converted into T3 in the specific tissue via enzymatic reactions (Schroeder and Privalsky, 2014). T3 function has been mainly studied in amphibians, where it controls the *de novo* growth and differentiation of tissues crucial for adult life, such as limbs, but also the remodeling and maturation of existing tissues, such as the intestine (Furlow and Neff, 2006). Teleost fish also undergo metamorphosis during their development (McMenamin and Parichy, 2013). In the zebrafish, even though it is phenotypically more subtle than in

Xenopus, morphological changes occur between 5dpf and approximately 1mpf, a stage at which small juveniles look like adult fish (Parichy et al., 2009). Significant changes in the body shape, development of adult fins, and formation of adult pigments patterns, as well as maturation and remodeling of several organs such as the lateral line, the gut, or the kidney appear during this period (McMenamin and Parichy, 2013). However, it remains unknown whether some events also affect brain development. Interestingly, we report that most of lateral pallium formation occurs between 5dpf and 1.5mpf, corresponding approximately to the metamorphosis period, with a majority of neurons that seems to be formed after 15dpf. Analysis of the T3/T4 ratio revealing the activity of T3 generation during zebrafish development indicates that a peak of T3/T4 is visible at around 15dpf and declines gradually up to 20dpf but reaches a low plateau only at around 1.5mpf (Chang et al., 2012). Thus, it might be that thyroid hormone and metamorphosis play some role in the development of the lateral pallium (Figure 37B). In the mouse, the expression pattern of TR receptors revealed that, whereas TRα1/α2 are broadly expressed in the developing brain, TRβ1, weakly expressed up to E17.5, is localized in the hippocampus at least up to E19.5 (Bradley et al., 1992).

Interestingly, in the adult SEZ, it has been reported that T3, via TRα1, promotes neurogenesis via the emergence of Type C cells (transit-amplifying) and Type A (committed neuroblasts) by inhibiting Sox2 expression (López-Juárez et al., 2012). Moreover, mice mutant for TRα1 display reduced hippocampal neurogenesis and memory impairment (Kapoor et al., 2010; Venero et al., 2005), indicating a role for thyroid hormone in activating adult neurogenesis.

It would be thus interesting to investigate the expression pattern of TR receptors in the zebrafish and determine whether some of them start to be expressed in lateral progenitors at the neurogenic switch. Moreover, if this hypothesis is true, functional inhibition of thyroid hormones activity or synthesis (with 6-n-propyl-2-thiouracil , methimazole or amiodarone) should disrupt neurogenesis in the lateral pallial VZ.

1.2.3 Progenitors adopting *de novo* neural stem cells features in the adult lateral VZ

The lateral pallial VZ originates from *her4*-negative/*her6*-positive/*her9*-positive progenitors located in the roof plate. However, we report that the lateral neural stem cells, ie. *her4*-positive RGCs, are generated continuously during life, even after the late juvenile stage. Indeed, long-term lineage tracing of cells expressing *her4* at 1.5mpf indicates that the vast majority of the *her4*-positive lateral NSCs are generated before 1.5mpf, but we observed a clear territory in the postero-lateral edge of the pallium that is not generated by the *her4*-

positive population at 1.5mpf and that continues to grow. This territory contains both RGCs, neurons and NE cells expressing *her9* and *wnt3a*, two features present in the embryonic progenitor pool at the origin of the lateral pallium. We interpreted this result as a possible growing zone present in the posterior pallium that, at least up to 8mpf, keeps on generating RGCs *de novo*. In agreement with this hypothesis, we observed in this region the same steps of progenitor maturation than during development: *her9*-positive/*her4*-negative NE progenitors abutting *her4*-positive NE progenitors, then *her4*-positive RGCs, suggesting that this corresponds to a maturing progenitor region with progenitors arranged in a maturation series (Figure 37A). However, lineage tracing of *her9*-positive cells in the adult would be necessary to confirm this hypothesis, and to precisely determine the hierarchy existing among the progenitor population in this region.

Interestingly, other neurogenic regions contain NE cells postulated to act as NSCs in the zebrafish as well as in the medaka adult brains (Alunni et al., 2010). First, NE cells are present at posterior levels in the periventricular gray zone (PGZ) of the optic tectum (Chapouton et al., 2006; Ito et al., 2010). Some of these cells are slow-proliferating progenitors (Alunni et al., 2010; Chapouton et al., 2006; Ito et al., 2010), participate in neurogenesis in the optic tectum, and it has been proposed that they produce both glia and neurons (Alunni et al., 2010; Ito et al., 2010); but whether neuronal production goes through a glial state remains to be clarified. Second, at least part of them express the non-canonical *her* gene *her5* (Chapouton et al., 2006). This gene is already present in the embryo and characterizes the “progenitor pool” at the midbrain-hindbrain boundary, suggesting that, like for the lateral pallium, some cells already present in embryonic “progenitor pools” are maintained during development and participate in adult neurogenesis. It will be important to perform the lineage tracing of embryonic *her5*-positive cells, to determine the molecular changes involved in the transition between NE and RGCs, as well as whether *her4* is involved in adult neurogenesis in the adult optic tectum, to determine whether the process involved in the maturation of lateral pallial progenitors is conserved in this other brain region.

The zebrafish adult retina also displays neurogenic as well as regenerative capacities (Lenkowski and Raymond, 2014) that rely on two types of progenitors: NE cells located in the ciliary marginal zone (CMZ) of the adult retina, and Mueller glial cells (Lenkowski and Raymond, 2014). NE cells are involved in generating all retinal cells including the Mueller glial population, the latter is dedicated to rod lineage production during development and after lesion. The sequence of progenitor maturation is spatially segregated with retinal stem cells at the CMZ: mutipotent progenitors are found at the most peripheral location, abutting committed retinal progenitors, and finally, differentiating retinal cells (Lenkowski and Raymond, 2014). Interestingly, *her6* is expressed in both the CMZ and Mueller glia of both embryonic and adult retina (Bernardos et al., 2005; Raymond et al., 2006). Similarly, during

retinogenesis in Xenopus, *Hes4*, homologous to zebrafish *her9,* has been shown to control retinal neural stem cell proliferation and its expression is modulated by both the Wnt and Shh pathway (El Yakoubi et al., 2012), two pathways that we found also in the postero-lateral regions of the zebrafish pallium. This emphasizes the relevance of functionally testing whether these pathways activate *her6/her9* in the zebrafish lateral pallial NE cells.

Altogether, this information highlights that several actively neurogenic structures in the adult nervous system display features similar to the lateral pallium, with the maintenance of NE cells that express non-canonical *her* genes, this in the zebrafish but also in Xenopus. It would be interesting to determine whether, in these different structures, NE cells are involved in producing neurons directly and/or produce glial cells that generate neurons. To do so, short-term single cell lineage tracing would be necessary to appreciate the immediate progeny of NE cells.

In mammals, a population of non-glial neural stem cells, also called Type 2 cells, has been described in the dentate gyrus of the hippocampus (Mu and Gage, 2011), and the hierarchy between this population and the Type 1 RGCs remains unclear. Indeed, one hypothesis would be that they constitute the NSCs and produce neurons without going through a glial state (Suh et al., 2007). Interestingly, both Type 1 and Type 2 cells express *Hes5* (homologous to the zebrafish *her4*) and they are mainly found in proliferation (Lugert et al., 2010). This suggests that in the mouse as well, several distinct cell types could serve as aNSCs in the SGZ.

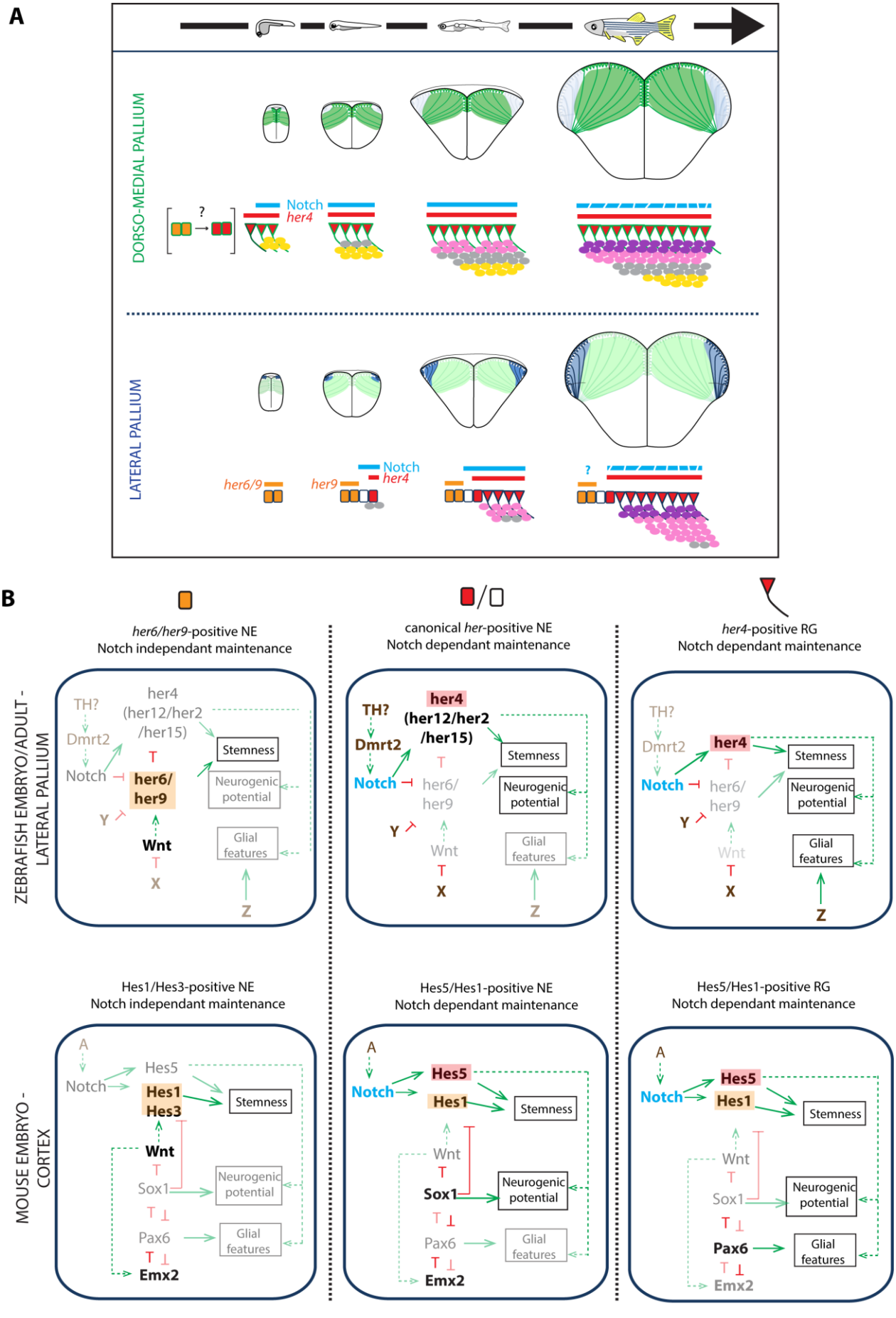
A
DORSO-MEDIAL PALLIUM
LATERAL PALLIUM
Notch
her4
her6/9
her9
?
B
her6/her9-positive NE
Notch independant maintenance
canonical her-positive NE
Notch dependant maintenance
her4-positive RG
Notch dependant maintenance
ZEBRAFISH EMBRYO/ADULT - LATERAL PALLIUM
TH?
Dmrt2
Notch
her4
(her12/her2
/her15)
her6/
her9
Y
Wnt
X
Z
Stemness
Neurogenic
potential
Glial
features
MOUSE EMBRYO - CORTEX
Hes1/Hes3-positive NE
Notch independant maintenance
Hes5/Hes1-positive NE
Notch dependant maintenance
Hes5/Hes1-positive RG
Notch dependant maintenance
A
Notch
Hes5
Hes1
Hes3
Wnt
Sox1
Pax6
Emx2

Figure 37: Working model of pallial progenitor maturation and pallium formation

(A) Summary of zebrafish pallium formation.

The dorso-medial neural stem cells (NSCs – green) originate from embryonic her4-positive (red bars) radial glial cells (green/red triangle). This population is maintained by the Notch pathway from embryonic stages onward (light blue bars). At adult stage, Notch signaling regulates NSCs maintenance by controlling proliferating rate (Alunni et al., 2013; Chapouton et al., 2010) (dashed light blue bars). Whether the her4-positive RG state is preceded by her4-positive neuroepithelial (green/red rectangle) and/or her6-positive neuroepithelial (green/orange rectangle) progenitors remains to be determined. The dorso-medial population generates neurons that pile up in the parenchyma lifelong from embryonic stages onward (embryonic neurons – yellow; larval neurons – grey; juvenile neurons – pink – adult neurons – purple), and progressively formed first the central pallium and then the medial and dorsal pallium.

The lateral neural stem cells (NSCs – blue) originate from her6/her9-positive (orange bars) neuroepithelial cells (blue/orange rectangle) that stay lifelong at the postero-lateral edge of the pallial VZ. Contrary to the dorso-medial population, these cells are not maintained by the Notch pathway during development and it still might be the case in the adult, but produce from larval stages onward Notch-sensitive (light blue bars) neuroepithelial cells (blue/white and blue/red rectangles) that start to express her4 (red bars), and further become radial glial cells (blue/red triangles). The production of her4-positive progenitors by the her6/her9 progenitors is continuous during development up to at least 1.5mpf and is likely still present in the adult. The lateral neuronal production is correlated with the start of her4 expression and thus the first lateral neurons are generated at larval stages (grey) but most of the lateral pallium is built at juvenile stages (pink), and neurons are still produced in the adult (purple).

(B) Comparison of the progenitor sequence visible during zebrafish lateral pallial development and at the postero-lateral adult VZ (upper panel) and mouse corticogenesis (lower panel).

her6/her9 are expressed in embryonic neuroepithelial cells (blue/orange rectangle) and this expression might be under the control of Wnt signaling as Wnt3a/Wnt8b are expressed in the same region. During mouse cortical development, expression of Hes3/Hes1 is Notch-independent at early stage and Wnt could be as well responsible for initiating Hes genes in this population. Moreover, Wnt activate Emx2 expression, a factor promoting neuroepithelial cells and symmetric divisions, but whether emx2 plays also this role in the zebrafish could be interesting to investigate. The expression of non-canonical (not activated by Notch) Hes/her genes is involved in maintaining the stemness of both mouse and zebrafish early progenitors.

In the zebrafish, the activation of the Notch pathway in the lateral neuroepithelial progenitors might be regulated by Dmrt2, already shown to promote telencephalic neurogenesis (Yoshizawa et al., 2011), and the latter could be activated by systemic cues such as the peak of thyroid hormon (TH) occurring at metamorphosis (around 5dpf). Once Notch maintains the early progenitors, it might participate in her6/9 downregulation and activate her4 (blue/red rectangle) or other canonical (Notch-sensitive) her genes such as her12/her2/her15 (blue/white rectangle), triggering a change in the set of genes involved in stemness maintenance. This change is also present in the mouse cortex as Hes3 is downregulated upon Notch activation, however, Hes1 expression is still present but becomes Notch sensitive (Kageyama et al., 2008). Moreover, in addition to Emx2, Sox1 is expressed in neurogenic neuroepithelial progenitors and promotes symmetric neurogenic divisions in this population by inhibiting Pax6 (Suter et al., 2009). Whether a network similar to Sox1/Pax6/Emx2 is involved in the zebrafish lateral progenitors in triggering changes in Wnt activation, division mode, and/or the transition between NE cells and RGCs is not known.

Hes5/her4 promotes neurogenesis in progenitors but whether it activates neuronal production or confers neurogenic capacity to the cells remains to be determined.

Finally, RGCs emerge from NE progenitors and this change is occurring in the mouse cortex via Pax6 expression that promotes also asymmetric cell divisions. Once again, what control the emergence of glial phenotype in the progenitors remains unknown in the zebrafish. But Hes5/her4 might be involved directly or indirectly in maintaining this glial phenotype in the progenitors.

2 Zebrafish pallium construction and homologies

Embryonic progenitors contribute to the generation of adult organs by producing differentiated cells that will possess one or several functions. In the mammalian brain, embryonic neural progenitors mainly generate neurons during development, but also produce glial cells such as oligodendrocytes or astrocytes, in a very regulated manner (Kriegstein and Alvarez-Buylla, 2009). Analyzing the progeny of the neurogenic neural progenitors during zebrafish pallium development allowed us to have an overview of how the pallium is built during development and the distribution of the embryonic and juvenile-born neurons. In a first part, we will discuss the general organization of the zebrafish neuronal pallial compartments and compare it with what we know on how the mouse pallium is organized. Second, the information on how the pallium is built will give us cues on homologies between the different pallial regions in zebrafish and mouse, as well as on the contribution of developmental processes to the generation of the everted zebrafish pallium.

2.1 Zebrafish pallium construction

2.1.1 Medio-lateral neurogenic gradient

As a first approach to study pallial construction, we reanalyzed at adult stage the lineage tracing of progenitors expressing *her4* at different time points during development; since we used a ubiquitously expressed reporter, it allowed us to analyze the neuronal progeny of the neurogenic progenitors from very early embryonic to late juveniles stages. We were able to approximate the developmental stage at which pallial neurons were generated during development and we concluded first that the most central pallium contains the oldest pallial neurons. By comparing these results with the lineage tracing of the *TP1Glob*-positive cells in the *TP1Glob:CreERT2* fish line, we determined that this region is mainly built by the progenitors generating the aNSCs of the medial pallium. The central neurons are mainly generated up to 5dpf; and later, the same pool of progenitors generates the dorsal and medial neurons. Most of the lateral pallium is generated between 15dpf and 1.5mpf illustrating a third wave in the generation of pallial neurons. Interestingly, it highlights that a centro-medial to lateral wave in the onset of neurogenic activity exists in the zebrafish pallium, suggesting that pallial neuronal production is tightly regulated. In mammals, a similar wave of neurogenesis has been reported, however, it corresponds to the exact opposite orientation with a lateral to medial neurogenic gradient (Anthony et al., 2004). But due to the everted structure of the zebrafish telencephalon, the pallial neurogenic gradient appears similar in mouse and zebrafish.

2.1.2 “Layers” and nuclei in the zebrafish pallium

The second piece of information resulting from this lineage is the temporal organization of the generated neurons. Contrary to the mouse cortex in which the neurons are organized following an “inside-out” sequence, in the zebrafish, they pile up in the pallium under the growing germinal zone, reflecting the temporal neurogenic activity of the progenitors. Even though no particular “layers” as in the mouse cortex are distinguishable in the zebrafish pallium, very little cell mixing seems to occur within the neurons generated at different time points during development, and this reflects that, despite a differently positioned ventricular zone, the neurons are organized in the same way in mouse and zebrafish with the oldest neurons “ventrally” positioned whereas the youngest neurons are “dorsally” located. Almost all the mouse pallial areas are more or less layered (Medina and Abellán, 2009). The isocortex contains six layers and this organization is due to two phenomena, the radial migration of the neurons along the RG processes, and the action of the Cajal-Retzius neurons (Borello and Pierani, 2010). The latter heterogeneous neuronal population is transiently present in the developing pallium as these neurons mostly disappear in the first two post-natal weeks (Kirischuk et al., 2014). They provide a “stop” signal via the production of Reelin protein in the marginal plate to the newly generated neurons by promoting their detachment from the RG cells (Honda et al., 2011), thus determine their final position within the developing cortex. In addition to Reelin production, recent studies have shown that they also guide neurons via cell-cell contacts mediated by Cadherin and Nectin proteins (Gil-Sanz et al., 2013). The organization of the neuronal compartment of the zebrafish pallium suggests that after birth the neurons stay close to their site of generation while the germinal zone is shifted dorsally due to the addition of new neurons. Reelin expression has been documented in the dorsal pallium all along zebrafish development (Costagli et al., 2002; Imai et al., 2012); however, whether it has a role in positioning pallial neurons remains unknown and the presence of Cajal-retzius cells during zebrafish pallial development has not been reported so far -but no lineage tracing experiments were performed-. In the mouse, Cajal-retzius cells are produced at the level of pallial organizers, including the cortical hem (Bielle et al., 2005). It is important to mention that, in the pallial region where Reelin expression is found during zebrafish development, neurons derive from *her4*-positive cells. Thus the dorsal *wnt3a*-expressing cells (considered as the cortical hem-like structure) would not be at the origin of dorsal Reelin-positive neurons. However, the anti-hem (pallial-subpallial boundary) and commissural septum of the mouse brain are sources for Cajal-retzius neurons as well, and both structures express Dbx1 in the mouse (Bielle et al., 2005). As previously mentioned *dbx1* is not expressed in the very early stage of development in the zebrafish telencephalon but its late expression pattern could be interesting to investigate; if expression is found in the pallium, lineage tracing experiments of *dbx1*-positive cells could help determine whether

some neurons similar to Cajal-retzius cells (at least from a developmental point of view) could be present in the zebrafish pallium. It is interesting to note that in the absence of Reelin in the mouse, the pallium organizes in the reverse way, in an “outside-in” manner, like the zebrafish pallium does (Magdaleno et al., 2002), indicating that Reelin expression in the zebrafish dorsal pallium could play another role than guiding neurons during development.

The layers of the mouse cortex are characterized by specific types of neurons displaying particular output and input connections, thus a specific function. For instance, neurons residing in the deep layers project mainly toward the thalamus, the midbrain and the spinal cord, whereas neurons in the upper layers (II and III) mainly display intrapallial connections. The emergence during development of these neuronal layers is temporally regulated with the deep layers generated first from the VZ, the layer VI at E12.5 and the layer V at E13.5, and later the upper layer from the SVZ, the layer IV at E14.5 and the layer III and II at E15.5 (Molyneaux et al., 2007). Large scale studies have identified plenty of factors expressed in one or more cortical layers, suggesting that cortical layer specification is very complex and relies on combinations of factors (Molyneaux et al., 2007). As an example, *Otx1* is expressed in both the VZ and deep layer neurons prior to and during layers V-VI generation (Frantz et al., 1994; Sancini et al., 2001), and *Cux1* and *Cux2* are expressed in the VZ, the mitotically active cells of the SVZ and in upper layer neurons (Nieto et al., 2004; Zimmer et al., 2004). *Pax6* expression has been shown to be necessary for the emergence of the upper cortical layers (Nieto et al., 2004; Zimmer et al., 2004), whereas its absence does not impair the production of *Otx1*-positive neurons of the layer V (Stoykova et al., 2000; Tarabykin et al., 2001). On the contrary, *Er81*, a layer V-specific projection neurons marker, seems to be a target of Pax6 (Tuoc and Stoykova, 2008), suggesting that the differential sensitivity of the cortical layers markers to patterning signal could influence temporal expression of such markers.

In the zebrafish adult pallium, neither the expression pattern of genes orthologous to the mouse cortical layers markers, nor morphology and connectivity have been thoroughly investigated yet. Adult expression analysis of the deep cortical layer marker, *Tbr1*, reveals that it is largely present, but without a particular “layered expression” as it is expressed in most of the dorsal pallium (Ganz et al., 2012). Thus, despite the organization of the zebrafish pallium in a “temporal” gradient, it seems to be “functionally” organized in a non-laminar manner. This expression pattern is much more related to the situation in the chick pallium, in which expression of some cortical layers markers define “regions” within the pallium rather than layers (Suzuki et al., 2012). However, whether these regions represent the functional equivalent of the cortical layers remains to be determined.

Finally, based on clonal and brainbow analyses, we report that, compared to the lateral zebrafish pallium, the dorso-medial compartment derives from a large number of embryonic neural progenitors at 1dpf. However, it remains to be determined whether, as it has been suggested to occur in the cortical progenitors, one single progenitor is able to generate different types of neurons along life. Long term clonal analysis coupled with a neuronal characterization would be necessary to investigate this.

Despite the apparent "non-organized" structure of the zebrafish pallium, the distinct spatio-temporal neurogenic activity of different progenitor populations correlate with the formation of different pallial nuclei and/or areas, and this could be related to the emergence of distinct complex behaviors depending on the developmental stage. For instance, a recent study of the lab has shown that neurons born at juvenile stage, rather than embryonic or adult born neurons, respond to D-amphetamine injections or drug-seeking in the 6mpf-old adult (von Trotha et al., 2014), suggesting that this behavioral output might not be obtained with larvae. Moreover, we report that the lateral pallium growth starts late during development. This region has been proposed as homologous to the mouse hippocampus (this point will be detailed later – see section 2.2.2) and interestingly timing of lateral formation is correlated with the appearance of learning capacities in the juveniles (Valente et al., 2012). Thus, the temporal pallial neuronal generation may have an impact and needs to be taken into account for further behavioral assays.

2.2 Zebrafish pallium regionalization and homologies

2.2.1 Zebrafish pallial subdivisions regarding embryonic origin and eversion

Previously, fish pallium subdivisions were defined as the medial pallium (Dm), the dorsal pallium (Dd), the central pallium (Dc), the lateral pallium (Dl) and the posterior pallium (Dp), with only the central pallium that would not comprise an adult ventricular zone (Braford, 2009). Our study based on direct genetic lineage tracing reveals a clear compartmentalization of the zebrafish pallium regarding its embryonic origin, but this compartmentalization diverges from the existing view of pallial regionalization. Indeed, we could delimit different pallial compartments, corresponding to a progenitor subopulation and its progeny. First, we defined the medial pallium that comprises Dm and most of the Dc domains. This compartment derives from the most medial early neurogenic progenitors (*TP1Glob*-positive/*her4*-positive progenitors) at 1dpf. Second, we defined the dorsal pallium, which comprises the most lateral part of Dc and the entire Dd domain. It derives from the most dorsal population of the early pallial neurogenic progenitors (*TP1Glob*-negative/*her4*-

positive progenitors) at 1dpf. And finally, the lateral pallium corresponding to Dl and Dp domain and that derives from the embryonic roof plate at 1dpf.

The ventricular portion of the Dm and Dd domains are thus the ventricular zone that built the Dc domain. As previously mentioned, no adult-born neurons seem to reach the Dc domain as neurons pile up in the parenchyma. But interestingly, in regenerative conditions, adult *her4*-positive RGCs has been shown to contribute to the regeneration of the central pallium after a stab lesion, indicating that the capacity to form neurons composing Dc is present in the adult pallial progenitors (Kroehne et al., 2011); it would be interesting to determine whether only dorso-medial RGCs participate in Dc regeneration.

Moreover, by performing long-term clonal analyses, we never observed any obvious central clones not connected to the ventricular zone, indicating that Dc with Dm and Dd domains share the same pool of progenitors and that Dc has a “ventricular” portion. But contrary to the theory already proposed by Mueller (Mueller et al., 2011a), it seems that this ventricular region is larger than only the most anterior dorsal pallium, and that it is devoted to generating Dc neurons only at early stages.

The other region of the zebrafish pallium is the lateral pallium comprising the Dl and Dp domains. The theory proposed by Wullimann and Mueller concerning the origin of the Dp domain postulated that Dp was migrating from the Dm region during development (Mueller et al., 2011b; Wullimann and Mueller, 2004). In contrast, we found that Dp and Dl are sequentially produced during development by the lateral embryonic progenitor pool, Dp being the latest pallial region to be formed in the zebrafish, and that continues to be formed during adulthood. It is interesting to note that the physical boundary between Dp and Dl is not clearly visible by simply lineage tracing of neurogenic progenitors. This emphasizes the strong relationship between the two domains, thus questioning whether it makes sense, regarding their embryonic origin, to separate them. However, it is interesting to note that only the most postero-lateral pallial region, included into the Dp domain, continues to grow at adult stage highlighting that it corresponds to a particular region of the lateral pallium.

2.2.2 Homology between the mouse hippocampus and the zebrafish lateral pallium

Determining the embryonic origin of the different pallial territories give us also information on homologies of the region between different species, as homologous structures derive from the same embryonic area. Most of the different theories of homology between mouse and zebrafish pallial regions consider that the zebrafish lateral pallium is homologous to the mouse hippocampus (medial pallium). However, these theories exclude Dp, mainly considered as homologous to the mouse piriform cortex, due to the olfactory inputs present

in this region (Braford, 2009). As already mentioned, we found that Dp corresponds to the ventro-posterior part of the lateral pallium and seems to derive from the same embryonic population as Dl even though it continues to grow in the adult. This was already suggested by Nieuwenhuys who noticed that the Dp domain is attached to the tela-choroida, the thin layer of cells that covers the ventricle (Nieuwenhuys, 2009). Nieuwenhuys considered this region as the ventral part of the lateral pallium, and postulated the entire lateral pallium to be homologous to the mouse hippocampus (Nieuwenhuys, 2009). Several arguments concerning neurogenic activity and timing of development, already discussed here, support that the lateral pallium of the zebrafish does host the region homologous to the hippocampus. Indeed, we mentioned that it develops late during development, mostly at a caudal level, close to a "cortical-hem" like structure, and contains non-glial *her4*-positive cells in its adult VZ, one feature already reported in the SGZ of the dentate gyrus. Moreover, lesion experiments performed in the goldfish in the lateral pallium impair the encoding of the geometric information of environmental space, a typical function of the hippocampus (Vargas et al., 2006), and hodology (neuronal connections) seems to support this theory (Northcutt, 2006). Finally, it is interesting to note that behavioral analyses indicated that learning in the zebrafish starts late during development, at around 20dpf, and reaches adult performances at around 1.5mpf (Valente et al., 2012), the time window corresponding to the formation of the lateral pallium. All these arguments strongly converge toward the homology of at least some parts of the lateral pallium (Dl and/or Dp) with the mammalian hippocampus.

However, analysis of markers specifically expressed in the adult mouse hippocampus revealed that only few genes present in the mouse hippocampus are expressed in the zebrafish lateral pallium. Analyses of expression of *pcdh1a/b*, *dusp5, er81* (Lein et al., 2004) and *prox1a* (Pleasure et al., 2000), expressed in the mouse hippocampus, indicate that only *pcdh1a/b* and *dusp5* are present in the lateral pallium (Figure 38) whereas *er81* and *prox1a* are not expressed (not shown). Interestingly, a recent study has compared the transcriptome of the mouse and chick hippocampus and revealed that *Dusp5* and *Pcdh1* are also present in the chick hippocampus (Belgard et al., 2013), but also revealed that very few genes display conserved expression between the mouse and chick hippocampus (Belgard et al., 2013); as an example, *neuroD* is not present in the chick hippocampus whereas it is expressed in the mouse. Thus, it is important to keep in mind that comparison of gene expressions at the adult stage between different species is not the best criteria and cannot alone define homology between two structures. In the zebrafish, it is interesting to note that *lhx2b* (Li and Pleasure, 2007), *neuroD* (Pleasure et al., 2000) and *tbr2* (Kimura et al., 1999) expressed during mouse hippocampus development are present in the lateral pallium as well (Figure 38), and could highlight maturing hippocampal-like cells. But expression of these

markers were analyzed at 1mpf and 3mpf and it would be interesting to determine whether they would be expressed earlier during the first phase of the lateral pallium development, such as 15dpf.

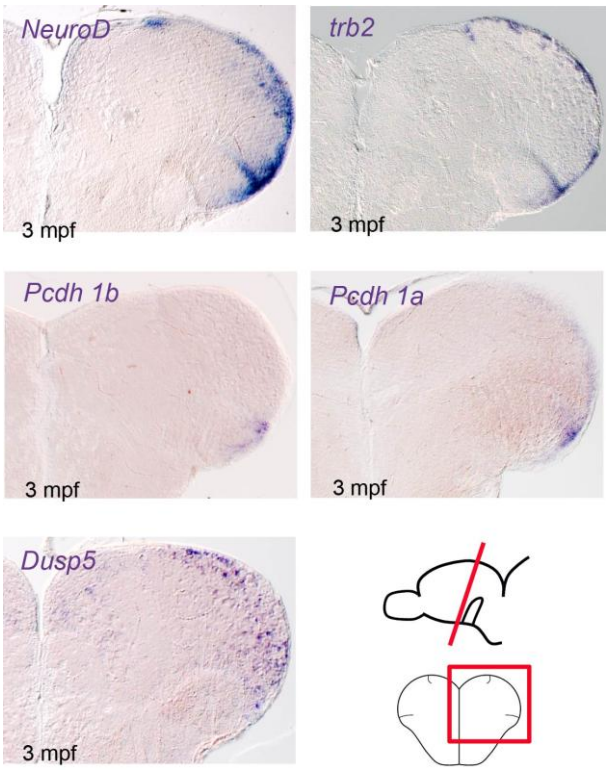

Figure 38: Expression pattern of genes orthologous to mouse hippocampal markers in the lateral domain of the zebrafish pallium (from S.Galant and I.Foucher)

2.2.3 Homology of other pallial territories between mouse and zebrafish

Concerning the different theories regarding the homology between Dm, Dd and Dc to the different mouse pallial regions, the medio-lateral gradient of development of the pallium and the absence of massive migration within the territory indicate suggest that the homologous pallial regions in mouse and zebrafish are organized in the opposite way along the medio-lateral axis.

The region homologous to the mouse lateral pallium would thus be located in the most medial pallium in the zebrafish, and we recenty showed in the lab that the Dm region seems to display some of the functions attributed in mammals to the amygdala (von Trotha et al., 2014).

Whether it exists a ventral pallium in the zebrafish is still under debate. The ventral pallium is defined by the *emx1*-negative/*tbr1*-positive pallial territory and generates part of the claustro-amygdaloid complex in the mouse (Puelles et al., 2000). It would be interesting to compare the lineage tracing of *emx1*-positive cells in the zebrafish with the lineage of *her4*-positive progenitors to determine whether a dorso-medial region of the zebrafish pallium does not derive from *emx1*-positive cells and would thus correspond to the zebrafish ventral pallium. In any case, it should be located in the most medial part of the zebrafish pallium.

The equivalent to the mouse dorsal pallium (generating the isocortex) remains unclear, but our experiments suggest that it should be located between the lateral and medial pallium. A recent study has indicated that this region performs some cortical functions such as the association of information (Aoki et al., 2013). Moreover, as previously mentioned, Reelin is present in this region during development (Costagli et al., 2002; Imai et al., 2012). The analysis of cortical markers should further help define this region even though, as for the hippocampus, genes expression is not the best criterion: for example, it has been shown that genes expressed in layer IV of the mouse cortex are highly different from the ones present in the chick nidopallium, considered as homologous to the cortex (Belgard et al., 2013). This emphasizes that structural markers analysis must be completed with functional assays, for instance based on neuronal ablation combined with specific behavioral assays, to be able to complete hypotheses of homology.

2.2.4 Late developmental processes participate in pallial eversion

The zebrafish pallium, contrary to the mouse pallium possesses an everted structure that positions the ventricular zone at the surface of the pallium and an extended tela-choroida attached to the pallium that closes the ventricle. How this eversion process occurs during development is not completely understood. Here, we report that the formation of the lateral pallium participates in accentuating the everted structure.

The first sign of eversion arises before 1dpf with the opening of the anterior intraencephalic sulcus that laterally positions the dorso-posterior part of the telencephalon (Folgueira et al., 2012). Analysis of *her4* expression indicates that this region is indeed *her4*-negative. Our lineage tracing experiment reveals that the cells at the origin of the lateral pallium are located in the roof plate and correspond to a small population of cells that we interpret as being positioned at the boundary between the extended roof plate and the pallial *her4*-positive region.

The second step reported is a dorsal repositioning of the posterior ventricle and an expansion of the pallium up to larval stage. This is in accordance with our lineage tracing

results as we observed *her4* expression along the posterior wall of the embryonic telencephalon, and *her4*-positive cells are at the origin of the dorso-medial domain.
Finally, we report that the lateral pallium remains very small up to late developmental stage. We observed a massive amplification of the lateral pallium at late juvenile stages and we can consider this event as the third step of the pallial eversion.

3 General conclusion

The results of this PhD thesis provide the first map of the embryonic origin of the entire pallial germinal zone in the zebrafish adult telencephalon. We reported first that the aNSCs of the zebrafish pallium derive from two different embryonic populations that diverge regarding neurogenic activity, number of progenitors, *her* genes expression and Notch dependency. Despite these distinct features, however, both populations generate aNSCs that display the same properties, - i.e. *her4*-positive radial glial cells, even though they remain spatially segregated. By comparing with the mouse, we were able to show that the developmental processes governing pallial development are conserved among species, thus emphasizing the potential of zebrafish aNSCs as a model for stem cell biology.
Second, we demonstrated the existence of two modes of aNSCs formation with the first one based on amplification of embryonic and juvenile progenitors already displaying NSCs features, and the second one with *de novo* NSCs formation all along development and at adult stage.
Third, these experiments allowed us also to investigate the general organization of the pallium in terms of the generation of its different spatial domains, in some cases in relation with function. Given the limited cell migrations observed in the pallium, the location of an adult neuron reflects both the location of its generating aNSC and its generation timing. Further studies will be necessary to investigate the functional significance of such architecture, in particular by analyzing the neuronal subtypes produced over time by a given aNSC clone.
Finally, by identifying the embryonic origin of the different pallial regions, this work enabled us to complete the different theories regarding homologies between the different zebrafish and mouse pallial domains, and especially emphasize that the lateral zebrafish pallium is the structure homologous to the hippocampus.

CHAPTER IV: REFERENCES

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SUMMARY

Adult neural stem cells (aNSCs) are defined by their self-renewal and multipotency, which allow them to generate both neurons and glial cells in the adult brain. Contrary to mammals, the zebrafish brain maintains numerous neurogenic zones in the adult, among which the most characterized is the pallial ventricular zone. It is composed of radial glial cells serving as aNSCs. Which embryonic neural progenitors are at the origin of these aNSCs is still unknown. This work aims to determine the relative contributions of two embryonic neural progenitor populations, the «proneural clusters» (involved in embryonic neurogenesis) and the « progenitor pools » (characterized by a delayed neurogenesis), to the formation of aNSCs in the zebrafish pallium.

First, using genetic lineage tracing techniques, we were able to identify the embryonic neural progenitor population at the origin of a subpopulation of aNSCs located in the dorso-medial part of the pallium. The *her4:ERT2CreERT2* transgenic driver line, combined with pharmacological treatments inhibiting the Notch signalling pathway, allowed showing that neural progenitors giving rise to dorso-medial pallial aNSCs express the « *Enhancer of split »* *her4* gene, specifically expressed in « proneural clusters » from very early stages of development.

As a second step, clonal analyses as well as spatially controlled recombinations by laser highlighted that aNSCs of the zebrafish lateral pallium do not derive from *her4*-positive embryonic progenitors maintained by the Notch pathway, but from a restricted population of neuroepithelial cells located in the embryonic telencephalic roof plate. These cells display « progenitor pool » specific features, as for instance the expression of non-canonical *her* genes (independent of Notch signalling) such as *her6* and *her9*, the expression of components of signalling pathways such as Wnt, BMP, FGF, and a late neurogenesis onset. These progenitors progressively generate, from juvenile stages, the vast majority of the aNSCs of the lateral pallium. Most interestingly, a small population of these neuroepithelial cells persists in the postero-lateral pallium at adult stage and keeps generating *de novo* aNSCs of this brain region.

In addition to identifying the origin of pallial aNSCs in the zebrafish, this study also delivers information on the progressive maturation steps that embryonic progenitors undergo to generate aNSCs, and highlights similarities and differencies existing between the dorso-medial and lateral progenitors. Finally, this work also permits tracing the neurons generated by stem cells at different stages. This reveals for the first time the progressive formation of the different zebrafish pallial compartements, and allows appreciating their homologies with the mouse pallial regions.

RESUME

Les cellules souches neurales adultes (aNSCs) sont définies par des fonctions d'auto-renouvellement et de multipotence qui leur permettent de générer dans le cerveau adulte tant des neurones que des cellules gliales. Contrairement aux mammifères, le cerveau de poisson zèbre présente de nombreuses zones de neurogenèse adulte dont la plus caractérisée est la zone ventriculaire du pallium. Elle est composée de cellules de glies radiaires qui font office de aNSCs dans cette partie du cerveau. Quels progéniteurs neuraux embryonnaires sont sélectionnés pour être à l'origine de ces aNSCs reste mal connu. Ce travail a pour objectif de déterminer la contribution relative de deux populations de progéniteurs neuraux embryonnaires, les "clusters proneuraux" (impliqués dans la neurogenèse embryonnaire) et les "pools de progéniteurs" (caractérisés par une neurogenèse tardive), dans la formation des aNSCs du pallium de poisson zèbre.

Dans un premier temps, à l'aide de techniques génétiques de lignage cellulaire, nous avons pu identifier la population de progéniteurs neuraux embryonnaires à l'origine d'une sous-population des aNSCs située dans la partie dorso-médiane du pallium. Des expériences de lignage utilisant la lignée de poisson zèbre *her4:ERT2CreERT2* combinées à des traitements inhibiteurs de la voie de signalisation Notch nous ont permis de déterminer que les progéniteurs neuraux donnant naissance aux aNSCs du pallium dorso-médian expriment le gène « *Enhancer of split » her4,* qui caractérise les "clusters proneuraux", ce dès des stades très précoces du développement.

Dans un second temps, des analyses clonales ainsi que des recombinaisons spatialement contrôlées par laser nous ont permis de mettre en évidence que les aNSCs de la partie latérale du pallium de poisson zèbre ne proviennent pas de progéniteurs embryonnaires exprimant *her4* et maintenus par la voie Notch, mais d'une population restreinte de cellules neuroépitheliales situées dans la plaque du toit du télencéphale embryonnaire. Ces cellules présentent des caractéristiques spécifiques des "pool de progéniteurs", à savoir l'expression de gènes *her* non-canoniques (dont l'expression n'est pas dépendante de la voie de signalisation Notch) tels que *her6* et *her9,* l'expression de ligands de voies de signalisation telles que Wnt, BMP et FGF, et une neurogenèse tardive. Elles génèrent progressivement, à partir du stade juvénile, une grande partie des aNSCs du pallium latéral. De plus, une partie de ces cellules neuroépitheliales persistent dans le pallium latéral postérieur chez l'adulte et continuent de former *de novo* des aNSCs dans cette région du cerveau.

Outre la vision globale que cette étude nous a permis d'avoir sur l'origine embryonnaire de la totalité des aNSCs du pallium de poisson zèbre, elle a aussi délivré des informations sur les étapes de maturation progressive des progéniteurs embryonnaires pour former les aNSCs, et les similitudes et divergences qui existent entre la population dorso-médiane et latérale à ce sujet. Enfin, en traçant les neurones issus des cellules souches à différents stades, cette étude a pour la première fois mis en évidence la formation progressive des compartiments neuronaux du pallium de poisson zèbre, et ainsi permis d'apprécier les homologies de ces compartiments avec les régions du pallium de souris.

RESUME

Les cellules souches neurales adultes (aNSCs) sont définies par des fonctions d'auto-renouvellement et de multipotence qui leur permettent de générer dans le cerveau adulte tant des neurones que des cellules gliales. Contrairement aux mammifères, le cerveau de poisson zèbre présente de nombreuses zones de neurogenèse adulte dont la plus caractérisée est la zone ventriculaire du pallium. Elle est composée de cellules de glies radiaires qui font office de aNSCs dans cette partie du cerveau. Quels progéniteurs neuraux embryonnaires sont sélectionnés pour être à l'origine de ces aNSCs reste mal connu. Ce travail a pour objectif de déterminer la contribution relative de deux populations de progéniteurs neuraux embryonnaires, les "clusters proneuraux" (impliqués dans la neurogenèse embryonnaire) et les "pools de progéniteurs" (caractérisés par une neurogenèse tardive), dans la formation des aNSCs du pallium de poisson zèbre.

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